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Cover Photo

Top left: Rotala rotundifolia (Lythraceae) collected from wetlands; **Left middle:** Root colonization by AMF in rice plant; **Left Bottom:** Antagonism of lactic acid bacteria against Mucor sp and Curvularia sp. **Middle:** Fruiting body of Lepiota procera growing in nature in Poonch District, Jammu; **Right top:** Grey bright disease of Som plant; **Right middle:** Germinated spores of Pestalotiopsis disseminate; **Right bottom:** Antagonism of Acti-6 and B-3 against Fusarium oxysporum.

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Bioprospecting for Microbial Endophytes and Their Natural Products

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Abstract
Endophytes are microorganisms that reside asymptomatically in internal tissues of all higher plants. There is growing interest amongst the researchers about this group of organisms because these are promising sources of biologically active agents. They are potential sources of novel natural products for scope of utilization in the pharmaceutical industry, agriculture, and in environmental applications. Many researches have proven that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture and industry. So far, a great number of novel natural products possessing antimicrobial activities have been isolated from endophytes. It is believed that screening for antimicrobial compounds from endophytes is a promising way to overcome the increasing threat of drug resistant strains of human and plant pathogen. Antimicrobial metabolites isolated from endophytes belong to diverse structural classes, including: alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids. These would provide the opportunity to utilize endophytes as a new source for production of antibiotics.

Key words: Endophytes; Natural products; Life cycle; Bioprospecting; Biodiversity; Bioremediation

Introduction

The term 'endophyte' (Gr. *endon*, within; *phyton*, plant) was first coined by de Bary (1866). They are microorganisms that inhabit living healthy plant tissues without causing any apparent manifestation of symptoms, and live in mutualistic association with plants for at least some part of their life cycle (Bacon and White, 2000). Initially the term endophyte broadly included organisms from foliar pathogens to mycorrhizal root symbionts. Later, fungi producing visible disease symptoms were excluded from this category even though all pathogenic fungi penetrate the host tissue and exist endophytically (Carrol, 1986). The term "endophyte" has undergone transformations though there still is considerable disagreement as what constitute an endophyte. Endophytes are extremely ubiquitous and it is hypothesized that the vast majority of plant species in natural ecosystems (if not all of them) harbor endophytes (Rodriguez *et al.,* 2009). All the species of plants studied to date are expected to harbour at least one such organism, nevertheless, endophyte-plant relationships are not well understood. Endophytes belong to diverse groups of bacteria, fungi, actinomycetes etc. (Bandara *et al.,* 2006). The most frequently isolated endophytes are fungi (Tayung *et al.*, 2008). Endophytic fungi are estimated to be represented by at least one million species residing in plants (Dreyfuss *et al.*, 1994). Usually, the fungal endophytes belong to Ascomycetes, Deuteromycetes and Basidiomycetes (Petrini 1986, Dayle *et al.*, 2001, Rakotoniriana *et al.,* 2008). As many as 110 different fungal species have been isolated from their coniferous hosts (Tayung *et al.*, 2008). The class and species of the fungi depend upon the host plants they are associated with. Krabel *et al.*, (2013) have hypothesized that the environmental conditions probably trigger the wood associated endophytic fungi to change from a mutualist to a virulent parasite. Research on endophytes dated back to over one hundred years (Petrini, 1986). During this period, several aspects of endophyte biology were thoroughly studied, including the diversity, taxonomy, reproduction, host ecology and effects on the host (Saikkonen *et al.,* 1998). They produce secondary metabolites, enhance hardiness of host plants, Provides resistance to fungal diseases by producing antimicrobial compound *in situ*. Increase plant resistance to haribivores and enhance plant competitive abilities. Because natural selection favors the evolution of beneficial endophytic strains, several endophytes were found to secret secondary metabolites that protect plants against herbivore (Robert *et al.*, 2004), insect (Spiering *et al.*, 2005), pathogens (Arnold 2003), abiotic stress (Waller *et al.*, 2005)

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and thus, endophytes represent a promising source of novel, biologically active metabolites for pharmacological and agricultural applications (Dreyfuss *et al.*, 1994; Schulz *et al.*, 2002). They can be used as biocontrol agents (Waller *et al.*, 2005).

Biochemical research revealed that a wide variety of natural products can be obtained from endophytic microbes (Schulz *et al.*, 2002; Strobel and Daisy, 2003). Natural products from endophytic fungi were observed to inhibit many pathogenetic organisms including bacteria, fungi, viruses and protozoans. Idris *et al.*, 2013, isolated *Cladosporium* sp. *Aspergillus* sp from *Kigelia Africana*, which inhibit *Bacillus* , *subtilis, Staphylococcus aureus* and *Escherichia coli* (I. Z. D. > 20 mm). Cercosporin, an effective anti-parasitic agent, has been isolated from the endophytic fungus *Mycosphaerella* sp associated with the plant *Sychotria horizontalis* in panama (Moreno *et al.,* 2011). Many antiviral agents were reported from endophytic fungi; two novel compounds cytonic acid A and B have been isolated from the endophytic fungus *Cytonaema* sp.

These compounds are inhibitor of human cytomegalovirus (hCMV) protease (Guo *et al.,* 2000). Due to host-endophyte coevolution, some plants that produce bioactive natural products have associated endophytes that produce the same natural products (Tan *et al.*, 2001). Since the microbial sources of bioactive compounds are easier and more economical for large-scale production than plant sources, the discovery that rare, valuable plant products might also be produced by their endophytic microorganisms is of special pharmacological interest (Strobel *et al.*, 2003).

A famous example is the anticancer agent 'Taxol' that is found in yew tree species (*Taxus sp.*). Stierle *et al.* (1993) have isolated and characterized a novel taxol producing fungus *Taxomyces andreanae*, from *Taxus brevifo*lia. Thus, when searching for novel, endophyte-based drugs, a particularly fruitful approach would be to survey traditional medicinal plants for the bioactive metabolites that may be produced by their associated endophytes (Verma *et al,* 2007; Huang *et al.,* 2008; de Siqueira, 2011). This review, therefore, focuses on the biology of endophytic organisms, their evolution and bioprospecting for natural products.

Evolution of endophyte–plant symbioses

All fungi invading plant foliage have an asymptomatic period in their life cycle that varies from imperceptibly short period (e.g. pathogens) to a life time (e.g. *Neotyphodium* endophytes in grasses). The endophyte is found in the embryo of infected seeds. They grow in to emerging leaves as the seed germinates. They remain concentrated in the base of the plants and not in roots. The endophyte grow up the stem and in to the seed head of the reproductive plant. Researchers believe that Endophytes may have developed genetic systems to communicate between themselves and the host plant (Borges *et al.*, 2009). Another probability of their evolution is their long-term coexistence within their hosts which resulted in a co-evolutionary process enabling these organisms to acquire interesting capabilities, such as ability of some of them to synthesize biologically active substances similar to the secondary metabolites produced by their hosts (Wang and Dai, 2011). This feature if utilized with the help of biotechnology could solve many problems we are facing today. Endophytes have been recognized as outstanding sources of novel bioactive compounds (Strobel, 2003). Some produce volatile organic compounds that benefit host plants by providing additional lines of defense against pathogens (Macías-Rubalcava *et al.*, 2010). Morath *et al.* (2012) advocated that the small gas-phase molecules be utilized through biotechnology because of their ability to produce a broad spectrum of aromatic compounds, including pleasant VOCs having useful agricultural and industrial properties (Zhi-Lin *et. al.*, 2012). Endophytic fungus- grass associations are generally treated separately from parasitic, pathogenic and saprophytic interactions and are viewed as mutualistic associations. Benefits to the partners are rarely symmetric and conflicting selection forces are likely to destabilize them. Endophyte–host interactions are based on mutual exploitation. There are, however, unanswered questions like how genetic diversity of the fungus and phenotypic plasticity in fungal life history traits, genetic combinations between the fungus and host, and the fungus and host individually or in concert as a phenotypic unit, respond to the changing selection pressures.

All plants are infested with microbes and may be symptomless i.e. epiphytes or endophytes and represent balanced state of symbiosis or with

symptoms i.e. diseased. They live asymptomatically and intercellularly within plant tissues. Both fungi (most frequently isolated) and Bacteria inhabit the plants. Relationship with host may be symbiotic or mutualistic. Some believe they may be aggressive saprophytes or opportunistic pathogens. This state represents the majority. The second category represents unbalanced state of symbiosis and are termed as pathogens. In endophyte plant relationships endophytes gain i.e. get shelter and nutrients this costs plants nutrients and resources. The plant gains in terms of growth promotion due to enhanced nutrient uptake, increased tolerance to harsh environments e.g. drought tolerance, induced resistance to pests and diseases.

The majority of published works indicate that endophytic fungi can be regarded as plant-defending mutualists producing biologically active alkaloids responsible for the evolution of the endophytic life-style of these fungi (Clay and Schardl, 2002). It, however, appears that the key factors responsible for evolution of the endophytic life-style of fungi are more complex involving multi-species interactions, multiple levels of causation and multidirectional flows of influence, and are influenced by stochastic events, such as abiotic and biotic environmental conditions, that drive the life histories of coevolving fungi and host plants (Fig.1) (Saikkonen *et. al.,* 2004).

Over the past 20 year much has been studied about a unique symbiotic interaction

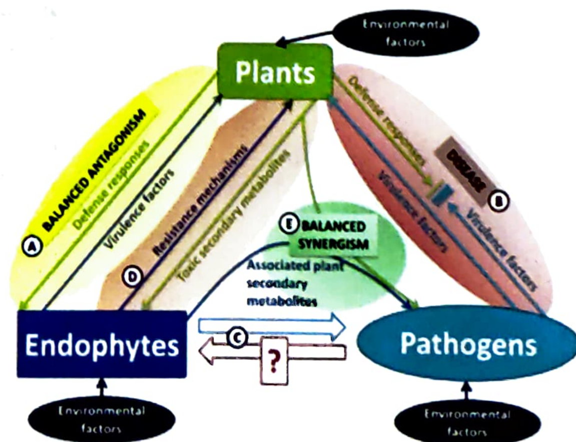

Fig. 1: Possible relationship between plant and microorganisms leading to evolution of endophytes (A) Balanced antagonism hypothesis (B) Plant disease caused by pathogenic fungi; (C) Endophyte-pathogen reciprocity. The question mark (?) indicates that this phenomenon might not be universal, and further research is necessary for verification. (D). Endophyte survival strategy; (E) Balanced synergism (Adapted from Saikkonen *et. Al.*, 2004).

between fungal endophytes and grasses. The fungi (Clavicipitaceae, Ascomycota) grow intercellularly and systemically in aboveground plant parts. Asexual endophytes of cool-season grasses that get vertically transmitted have been repeatedly derived from sexual species that abort host inflorescences. The phylogenetic distribution of seed-transmitted endophytes is strongly suggestive of cocladogenesis with their hosts and the molecular data suggest that many seed-transmitted endophytes are interspecific hybrids. Superinfection may result in hyphal fusion and parasexual recombination. Most endophytes produce one or more alkaloid classes that likely play some role in defending the host plant against pests. Hybridization may have led to the proliferation of alkaloid-production genes among asexual endophytes, favouring hybrids. The ergot alkaloid ergovaline, lolitrems, and lolines are produced by only a single sexual species, *Epichloe festucae*, but they are common in seed-transmitted endophytes, suggesting that *E. festucae* contributed genes for their synthesis. Asexual hybrids may also be favoured by the counteracting of the accumulation of deleterious mutations (Muller's rachet). Endophyte infection can provide other benefits, such as enhanced drought tolerance, photosynthetic rate, and growth. Estimates of infection frequency have revealed variable levels of infection with especially high prevalence in the subfamily Pooideae. Longitudinal studies suggest that the prevalence of seed-transmitted endophytes can increase rapidly over time. In field experiments, infected tall fescue suppressed other grasses and forbs relative to uninfected fescue and supported lower consumer populations. Unlike other widespread plant/microbial symbioses based on the acquisition of mineral resources, grass /endophyte associations are based primarily on protection of the host from biotic and abiotic stresses.

"Endophyte" is a generic term for any organism that lives inside of a plant, analogous to an epiphyte living on the plant surface. There has been semantic disagreement over usage of the term endophyte, with the suggestion that the word implies a mutualistic relationship that may not exist and that other words may be better (Wennstrom, 1994). For example, some plant pathogens like smuts may exist internally and asymptomatically within host plants for many years before they finally become evident.

Nevertheless, a large variety of heterotrophic organisms exist internally within plants, at least during part of their life cycle, without producing any disease symptoms. Most attention has been focused on fungal endophytes that exist in leaves, stems, and reproductive organs of host plants. The straight forward technique of surface sterilizing a leaf or leaf segment and plating it out on nutrient agar will generally result in the outgrowth of one or more fungi even if the leaf was asymptomatic. Asymptomatic, endophytic fungi may be ubiquitous in the plant kingdom, reviling insects in their species diversity (Carroll 1988; Arnold *et al.* 2000). But we know relatively little at present about the distribution and diversity of endophytes in different plant groups and plant communities, outside of a few well studied examples. Grass Endophytes Grass endophytes may constitute a monophyletic clade with the fungal family Clavicipitaceae (Ascomycota; Kul dau et al. 1997), but even with recent data it remains unclear if this is a monophyletic clade. Clavicipitaceous fungi include parasites of the grass family (and occasionally sedges) that can form pathogenic or mutualistic relationships with their hosts. Three of the four tribes infect only grasses or sedges, while the fourth tribe, Cordycipieae (genus Cordyceps), is pathogenic on insects or other fungi (Kuldau *et al.,* 1997). The tribe Clavicipeae (i.e., Claviceps) parasitizes a wide range of grasses where it forms infections of single grass florets and replaces the seed with individual sclerotia. These are the well-known ergot pathogens that produce toxic ergot alkaloids (Groger, 1972). The most diverse tribe is the Balansieae, consisting of several genera forming systemic infections of host grasses that also produce alkaloids (Bush *et al.*, 1997). One genus (*Epichloe*) has spawned a diversity of asexual forms (*Neotyphodium* species) that have radiated in association with cool-season grasses in the subfamily Pooideae (Schardl, 1996). Our primary focus is on these associations, reflecting the substantial research efforts devoted to this group. Growth in the Host Growth of clavicipitaceous endophytes in grasses exhibits several distinctive features. Growth is systemic throughout the aboveground tissues of their hosts (fig. 1A). Sparsely branched hyphae grow parallel to the long axis of plant cells in intercellular spaces where they likely subsist on sugars and amino acids released into the apoplast. Infections are perennial such that plants will remain infected throughout their life span; although sectoring and loss of infection in particular segments of host plants can be occasionally observed. During host flowering, the fungus grows into ovules and seeds or it proliferates to form a fruiting body. Molecular evidence suggests that most endophyte hosts are infected by only a single fungal genotype (Kover *et al.*, 1997; Meijer and Leuchtmann, 1999). Multiple infections can occur occasionally and may be highly significant because they afford the opportunity for fungal hybridization (Schardl *et al.,* 1994; Tsai *et al.,* 1994). Experimental inoculations with multiple strains show that all but one strain is eventually excluded at either the whole plant or tiller level (Wille *et al.,* 1999; Christensen *et al.,* 2000). In other endophytic associations with nongrass hosts, infections are typically highly localized and may consist of only a few epidermal cells. One leaf or one plant may be infected by dozens or hundreds of distinct fungal species (An *et al.,* 1992; Saikkonen *et al.*, 1998; Arnold *et al.,* 2000).

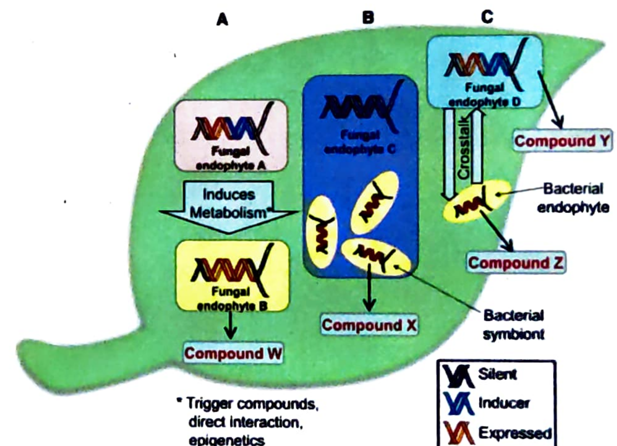

Fig.2 Communication sharing amongst different endophytic organisms leading to production of different molecules (A) Fungus-fungus crosstalk; (B) Fungus-bacterial endo-symbiont crosstalk (Adapted from Saikkonen *et. al.*, 2004)

Endophyte–host interactions are based on mutual exploitation

All fungi invading plant foliage have an asymptomatic period in their life cycle that varies from an imper-ceptibly short period (e.g. pathogens) to a lifetime (e.g. *Neotyphodium endophytes* in grasses). Endophytic fungus–grass associations are generally treated separately from

parasitic, pathogenic and saprophytic interactions and are viewed as mutualistic associations. However, endophyte–host interactions are based on mutual exploitation. Benefits to the partners are rarely symmetric and conflicting selection forces are likely to destabilize them. Unanswered questions are how (i) genetic diversity of the fungus and phenotypic plasticity in fungal life history traits, (ii) genetic combinations between the fungus and the host, and (iii) the fungus and host individually or in concert as a phenotypic unit, respond to changing selection pressures (Saikkonen *et al.,* 2004).

Although knowledge of the ecology, life history and phylogeny of endophytic fungi has accumulated rapidly during the past two decades, basic questions about the evolutionary origin, speciation and ecological role of endophytes remain largely unanswered [Clay *et al.,* 2002]. Although the term 'endophyte' has been controversial since it appeared [De Bary *et al.*, 1866, Petrini, 1991, Wilson, 1995], it has become synonymous with mutualism. However, recent studies show that the ecological role of even systemic grass endophytes can be complex and labile. Functionally, in terms of interactions with their host, different fungi are scattered throughout phylogenetic lineages [Clay *et al.*, 2002, Saikkonen *et al.*, 1998; Faeth *et al.*, 2002]. Moreover, defense of the host plant via endophyte mycotoxins, the most often cited mechanism of mutualism, discovered in agronomic grasses, seems rare in most native grass– and tree– endophyte interactions (Saikkonen *et al.*; 1998, Faeth, 2008; Ahlholm, 2002; Faeth, 2002). Nonetheless, the majority of published studies are still based on the conventional wisdom that endophytic fungi are plant-defending mutualists, with fungus-produced, biologically active alkaloids as key to the evolution of the endophytic life-style of these fungi (Clay, 2002). We propose that key elements for the evolution of the endophytic life-style of fungi are more complex, and involve multi-species interactions, multiple levels of causation and multidirectional flows of influence, and are influenced by stochastic events, such as abiotic and biotic environmental conditions, that drive the life histories of coevolving fungi and host plants.

Genetic diversity of the fungus and phenotypic plasticity in fungal life history traits

A number of pasture and turf grass species form mutually beneficial symbiotic associations with endophytic fungal species. Within the fescue grasses, diploid meadow fescue (*Festuca pratensis* Huds.) interacts with *Neotyphodium uncinatum* while allohexaploid tall fescue (*Festuca arundinacea* Schreb.) has been reported to associate with *Neotyphodium coenophialum* and two other morphologically distinct taxa (*Festuca arundinacea* taxonomic groups 2 and 3 [*Fa*TG-2 and *Fa*TG-3]). The evolutionary history of hexaploid tall fescue is complex, as part of a species group with varying ploidy levels and exhibiting distinct eco-geographical morphotypes. To evaluate both naturally occurring variation and host grass taxon specificity, diversity was determined in collections representing multiple meadow fescue and tall fescue accessions. Initial screening with a minimal set of endophyte-specific simple sequence repeat (SSR) genetic markers detected endophyte incidence in 33% of 701 tested accessions. Subsequent analysis identified N. coenophialum genotypes within continental and rhizomatous hexaploid and octoploid tall fescue [F. arundinacea sub sp. atlantigena (st.-Yves) Auquier] accessions. Festuca arundinacea taxonomic group 2 and FaTG-3 endophytes appeared to be restricted to Mediterranean hexaploid and decaploid tall fescue [F. arundinacea cirtensis (St.-Yves) Gamisans] hosts. Endophytes of meadow fescue were confirmed as belonging to N. uncinatum. This study has elucidated host specificity of fescue endophyte taxa and supported models for host–symbiont coevolution. A substantial number of candidate novel endophytes have been identified that are suitable for metabolic characterization and deployment by inoculation in fescue breeding programs (Ekanayake et al., 2011)

Lima *et al.,* 2012, reported thirty-nine endophytic fungi identified as *Colletotrichum* spp. associated with Brazilian pepper tree or aroeira (Schinus terebinthifolius Raddi. Anacardiaceae)in Parana state, Brazil. These endophytes were identified by morphological and molecular methods, using PCR taxon-specific with CaInt/ITS4, CgInt/ITS4, and Col1/ITS4 primers, which amplify specific bands in *C. acutatum, C. gloeosporioides lato sensu*, and *Colletotrichum boninensis*, respectively, and by DNA sequence analysis of the rDNA internal transcribed spacer region (ITS1, 5.8S, ITS2). We also assayed the

presence of dsRNA particles in *Colletotrichum* spp. isolates. Combining both morphological characters and molecular data, we identified the species *C. gloeosporioides*, C. *boninense*, and *C. simmondsii*. However, we found a high genetic variability intraspecific in *C. gloeosporioides* which suggests the existence of several other species. Bands of double-stranded RNA (dsRNA) were detected in three of thirty-nine isolates. Identity of these bands was confirmed by RNAse, DNAse, and S1 nuclease treatments for the isolates LGMF633, LGMF726, and LGMF729. This was the first study reporting these particles of dsRNA in *C. gloeosporioides.*

Phenotypic plasticity in fungi was observed on phenotypic changes in the colony morphology of the fungus *Aureobasidium pullulans* (Slepecky *et al.,* 2009). The variation in colony form is shown to depend on (i) the types of single carbon substrates (sugars and sugar alcohols) used in the growth medium, (ii) colony age, (iii) incubation temperature, (iv) light cycle and (v) substrate type. Expanding colonies grow in a developmental sequence that show synchronize growth phase shifts as well as unusual transitions from homogeneous to sectored, yeast to mycelial and giant to micro colonial growth forms. Epigenetic influences on phenotypic switches are suggested to be potential causes of form changes. The fungus *Aureobasidium pullulans* reversibly forms different types of colonies depending on the substrate and temperature on which it is grown, that is its environment. This property coupled with other natural attributes suggests that this microorganism could serve as a model for investigating a diversity of problems on the causes of phenotypic plasticity.

Fungi are notable for their ability to switch growth forms in response to environmental stimuli (Rayner and Coates 1987). Most likely fungi rely on the capacity to make these shifts to achieve survival, dispersal and reproductive advantages, and no doubt their success at these fundamental processes helps explain their recognition as a kingdom. The ability of fungi to alter forms and shift to different modes of living has been of interest to mycologists because of their importance to understanding fungal molecular biology, ecology and evolution as well as their utility in industry and their role in both infection and biological control. One fungus that assumes many different shapes (i.e. it is pleomorphic) and lives in a wide variety of habitats is *Aureobasidium pullulans*. This fungus has been recovered from diverse surfaces types, especially the phylloplane (Andrews *et al* 2002, McGrath and Andrews 2007, Andrews and Harris 1997, Woody *et al* 2007). Examples of other surface sources include glass (Schabereiter-Gurtner et al 2001), painted material (Shirakawa et al 2002), as well as rocks and marble (Urzı *et al* 1999, 2001). It is found in soil, freshwater and saltwater, ice (Zalar *et al* 2008) and is commonly recovered from the atmosphere (e.g. Shelton et al 2002, Lugauskas *et al* 2003, Griffin et al 2003, Samson et al 2004) and above (i.e. the Mir space station, Alekhova *et al* 2005). Unusual sources of *A. pullulans*, often as a contaminant, include for example samples containing ancient DNA (Hauf *et al* 1995), aviation fuel (Rauch et al 2006), spacecraft (La Duc *et al* 2003) and damaged nuclear reactors (Zhdanova *et al* 2000). *Aureobasidium pullulans* is involved as the principal colonizer initiating biodeterioration (e.g. plasticized polyvinyl chloride, Webb *et al* 2000) has been used as an indicator of environmental pollution (Deshpande *et al* 1992) and is implicated in human disease (Taylor *et al* 2005). The pleomorphic characteristic of fungi (Savile 1969) is also known as "phenotypic plasticity", that is the ability of any organism to respond to environmental signals by altering morphology, physiological state or behavior (West-Eberhard 1989). This ability is widespread among taxa and has been studied extensively primarily because of its importance to an organism's ability to survive and propagate. The function that describes the range of phenotypes produced by a single genotype in a suite of environments is called a "reaction norm" and is a concept generally adopted by geneticists studying evolution and ecology (Pigliucci 1996). Because over their lifetimes organisms occur in changing environmental conditions they are expected to have reaction norms that scale to the variable environment they inhabit and thus the individual is expected to be phenotypically plastic. What determines the shape of the reaction norm and how the change from one phenotype to another occurs are central questions in molecular, evolutionary and ecological genetics. Pigliucci (1996) discusses two broad approaches to studying phenotypic plasticity. One is statistical, which uses the tools developed by students of quantitative genetics. The major limitation of this method is that the assumptions underlying the

theory are often too simple and as a consequence inferences about genetic mechanisms can be unrealistic. The second approach is a mechanistic study of the genes involved in phenotypic plasticity. The initial phase of this approach is to use a genetic screen designed to detect plasticity genes or genetic networks involved in the transition from one phenotype to another. To facilitate this type of work model plants and animals, such as Arabidopsis thaliana or Drosophila melanogaster, often are employed as experimental organisms. Even though microorganisms have been used for studies in plasticity (Promislow 2005, Stomp *et al* 2008), their great potential for understanding the mechanism of phenotypic plasticity have not been generally recognized, especially in fungi (Jennings 1993, Andrews 1992, Bago *et al* 2004). Bacterial colonies growing on the surfaces in Petri dishes show differentiated structures that result from a complex series of morphological events. The geometry of bacterial colonies can be a consequence of swarming, chemotactic auto-aggregation, self-engineering, intercellular communication, nutrient gradients and stress (Shapiro 1995, Ben-Jacob and Levine 2006). Shapiro (1998) emphasized the need to consider a bacterial population as a multicellular organism with complex signalling systems that result in coordinated behaviours. These emergent phenotypes of single cells growing together and communicating affect survival, movement and reproduction, all of which can be beneficial and thus evolve. Pattern formation in bacteria, such as *Bacillus subtilis* (Mimura *et al* 2000), exemplify the degree of phenotypic plasticity that can occur in microorganisms grown in relatively simple culture conditions. In this case colony morphology show characteristics of "phase transitions" where there are abrupt changes from one morphologic type to another along nutrient and agar density gradients.

Genetic combinations between the fungus and the host

Even highly mutually beneficial microbial-plant interactions, such as mycorrhizal- and rhizobial-plant exchanges, involve selfishness, cheating and power-struggles between the partners, which depending on prevailing selective pressures, lead to a continuum of interactions from antagonistic to mutualistic. Using manipulated grass-endophyte combinations in a five year common garden experiment, we show that grass genotypes and genetic mismatches constrain genetic combinations between the vertically (via host seeds) transmitted endophytes and the out-crossing host, thereby reducing infections in established grass populations. Infections were lost in both grass tillers and seedlings in F1 and F2 generations, respectively. Experimental plants were collected as seeds from two different environments, i.e., meadows and nearby riverbanks. Endophyte-related benefits to the host included an increased number of inflorescences, but only in meadow plants and not until the last growing season of the experiment. Our results illustrate the importance of genetic host specificity and transgenerational maternal effects on the genetic structure of a host population, which act as destabilizing forces in endophyte grass symbioses. Genetic mismatches may act as a buffering mechanism against highly competitive endophyte-grass genotype combinations threatening the biodiversity of grassland communities (Axelrod *et al.*, 1981) and these mismatches should be acknowledged, particularly in breeding programmes aimed at harnessing systemic and heritable endophytes to improve the agriculturally valuable characteristics of cultivars (Bronstein, 1994).

Mutualistic interactions between microbes and plants are viewed as a ubiquitous cooperation conferring reciprocal benefits to the partners. However, even seemingly highly mutualistic interactions (e.g. between plants, mycorrhizal fungi and/or rhizobia) are inherently unstable, because reciprocal cooperation is based on mutual exploitation and thus costs and benefits are rarely symmetric to the partners (Axelrod *et al.*, 1981, Bronstein, 1994, Smith et al., 1997, Saikkonen,1998, Kiers et al., 2008, Saikkonen, 2004, Cheplick et al., 2009]. Consequently, microbialplant interactions, like any other biological interspecific interaction [Axelrod *et al.*, 1981, Bronstein, 1994, Smith et al., 1997, Saikkonen et al.,1998, Kiers et al., 2008, Saikkonen et al., 2004, Cheplick et al., 2009, Pellmyr et al.,1994, Herre et al.,1998, Stadler et al., 2005, Thompson et al., 2005, Sachs et al., 2006], involve selfishness, cheating and power-struggles between the partners, thus forming a continuum of interactions from antagonistic to mutualistic [Kiers et al., 2008],

with an occasional breakdown in mutualism [Sachs *et al.*, 2006]. The symbiosis between endophytes and grasses is generally considered to be a classic example of microbe-plant mutualism driving grassland communities [Omacini et al, 2001], as well as those food webs subsisting upon them [Omacini et al, 2001, Saikkonen et al, 2006]. The close link between endophyte fitness and its host grass is presumed to align the interests of both partners towards a mutually beneficial cooperation [Saikkonen et al., 2004, Cheplick et al., 2007, Saikkonen et al., 2006,, Clay et al., 2002], a view which seems to be supported by empirical evidence. In this highly integrated symbiosis, hyphae grow intercellularly and asymptomatically throughout the above-ground tissues of the host grass. Through growing into the developing inflorescence and seeds, the fungus is vertically transmitted from maternal plant to offspring. Evolutionary evidence of strictly asexual Neotyphodium and sexual Epichloe¨ endophytes suggests that such vertical transmission is concomitant with a reduced ability for contagious spreading by asexual or sexual spores and genetic host specificity (Clay et al., 2002). Because the fitness and distribution of a fungus largely depends on host fitness (Saikkonen et al., 2004), any mutualistic cooperation providing a selection advantage to the host plant also benefits the fungus. Conversely, reciprocal benefits from the fungus to the host plant, such as increased growth, resistance to biotic and abiotic stresses and enhanced competitive abilities (Saikkonen, 2005), further support the idea of endophyte-grass mutualism (Clay et al., 2002). Nevertheless, in most endophyte-grass interactions partner benefits and symbiotic dependence are asymmetric (Saikkonen, 2004). Symbiosis is essential for an endophyte because during its systematic growth the fungus subsists entirely on and within the host grass and vertical transmission via host seeds is the primary mode of fungal distribution (Clay et al., 2002). By contrast, the symbiotic relationship remains only conditional to the host plant, as plant fitness does not necessarily depend on the fungus (Saikkonen et al., 1998, Saikkonen et al., 2004, Saikkonen et al., 2006). In fact, in some environments symbiosis may even be maladaptive (Ahlholm et al., 2002, Faeth et al., 2003). For example, in endophyte species capable of sexual reproduction, the production of its fruiting body is costly to the host in terms of prevented flowering (Schardl *et al.*, 2004). Furthermore, in completely asexual endophyte strains, the adaptive value of symbiosis to the host grass appears to vary among fungal strains, being more pronounced in nutrient-rich environments (Saikkonen et al., 2006), as well as being dependent on plant-plant interactions in grassland communities (Clay *et al.*, 1999, Lehtonen *et al.*, 2015) and trophic interactions in food webs (Clay *et al.*, 1999, Rudgers *et al.*, 2008, Saari *et al.*, 2002, Saikkonen *et al.*, 2010). Accordingly, the infection incidence of grass species and populations appears to be highly variable spatiotemporally (Saikkonen *et al., 2000,* Jensen et al., 2004, Wei et al., 2006, Wali et al., 2007, Saari et al., 2009), reflecting how fungus and host alike respond to changing selection pressures, either individually or as a phenotypic unit (Saikkonen et al., 2004).Here, we use endophyte manipulation trials and a five year common garden experiment to test the importance of genetic compatibility to endophyte-grass symbiosis.

Genetic compatibility was examined in three transgenerational phases from the parental plant generation to those of the F1 and F2 generations; first at the initial encounter of the fungus and the grass, then in the success of the vertical transmission of the fungus to the vegetative propagules (tillers) and offspring of the host grass. The reasoning is that the asymmetric dependence of the endophyte and the host grass may lead to (Axelrod et al., 1981) host plant sanctions against less beneficial fungal strains in prevailing selective pressures and (Bronstein et al., 1994) the loss of the vertically transmitted fungus, which is continually confronted with new genetic combinations in the out-crossing host population. This is because the endophyte genotype remains unchanged in the plant lineage whilst plant genotypes are blended through recombination over time (Saikkonen et al., 2004). This could lead to a genetic mismatch between the fungus and the host, thus destabilizing the symbiosis and constraining the diversity of successful genotype-genotype combinations of the vertically transmitted endophytes and the host grasses.

Role of endophytes

Different works carried out so far regarding the role of endophytes in host plants indicate that

they can stimulate plants growth, increase disease resistance, improve plant's ability to withstand environmental stresses and recycle nutrients (Sturz and Nowak 2000; Strobel 2002; Johri 2006). Endophytes that reside in leaves and stems of plants contribute to the host's successful survival. The array of alkaloids and other chemicals synthesized by the endophytes endow the plant with more resistance to nematodes (worms), insect herbivores and livestock (Schulz et al. 2004). Besides these, endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan and Zou 2001; Strobel and Daisy 2003). Many of these compounds are bioactive and the range includes alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenols as well as some chlorinated compounds. There is a need to investigate fungal endophytes from medicinal plants because it has been hypothesized that these plants harbor some distinct and rare microbes that mimic the chemistry of their respective hosts and synthesize identical bioactive natural products or derivatives that are more bioactive than the one produced by the host. Strobel and Daisy (2003) have necessitated the need to study plants growing in unique environmental settings having ethno medicinal uses, extreme age or interesting endemic locations because they are expected to harbor novel endophytes that may produce unique metabolites having diversified applications. Many scientists believe that plants growing in lush tropical rainforests, where competition for light and nutrients is severe, are most likely to host the greatest number of bioactive endophytes than temperate parts of the worlds (Owen and Hundley 2004). The indigenous communities have been using medicinal plants in different ways for the treatment of various diseases, which in turn has resulted in scientific discoveries, with a wealth of literature on plant extracts and their biological activities. Wang et al. (2007) have demonstrated that the endophytes isolated from these plants are excellent producer of strong fungicidal and bactericidal metabolites.

Edophyte diversity

Great diversity of microbes are isolated from different healthy parts such as leaves, stem, fruits and roots of the ethnomedicinal plants (Ahmed *et al*, 2012). Majority of these endophytes isolated are fungi followed by bacteria and a few actinomycetes (Table 1). Ahmed *et al*, (2012) isolated a total of 5 endophytic fungal strains (LBBR01, LBBR02, LBBR03, LBBR04 and LBBR05) from Baru and screened them for antimicrobial activity by disk diffusion method. This test is accepted by the FDA (Food and Drug Administration) and it is established as standard by NCCLS (National Committee for Clinical Laboratory Standards). Caruso *et al.* (2000) isolated 150 fungal and 71 actinomycete endophytes from the internal tissues of woody branches, shoots and leaves of different plants of *Taxus baccata* and *Taxus brevifolia*. Arnold *et al.* (2000) isolated 418 endophyte morpho species from 83 healthy leaves of *Histeria concinna* and *Ouratea lucens* in a low land tropical forest of central panama, and proposed that tropical endophytes themselves could be hyperdiverse with host preference and spatial heterogeneity. Similarly, Jalgaonwala *et al.* (2010) isolated 78 bacterial and 142 fungal endophytes from aerial and underground parts of various medicinal plants. Teerayut *et al.* (2009) isolated 194 fungal endophytes from wild medicinal plants of Thailand. Santhosh *et al.* (2011) isolated 41 endophytic fungi from 195 samples of healthy leaves and stem of a red listed endangered medicinal plant *Coscinium fenestratum*.

Bioprospecting

Bioprospecting is defined as the systematic search for new sources of chemical compounds, genes, proteins, microorganisms and other products that have potential economic value present in our biotic resources, traditional knowledge often assist the bioprospecting process.

Problems that we face today include multi drug resistance, infectious microorganisms e.g. *Staphylococcus, Mycobacterium, Streptococcus* that have bcome ressitant to existing chemicals, appearance of diseases like AIDS, SARS etc, ancilliary infections due to weak immune system and infection by opportunistic pathogens like *Aspergillus* spp, *Crytococcus* spp, *Candida* spp. These infections are common in Immunocompromised patients. Protozoal and nematodal infections like maleria, trypanomiasis, filariasis etc. Environmental and health problems due to indiscriminate use of agrochemicals. Do

we need new medicines/agrochemicals to fight these problems? The answer is yes.The new chemicals, however, should have the characters like high affectivity, low toxicity, natural and minor or no environmental impact. Basis of modern medicines include combinational

Table 1: Different endophytic microorganisms isolated from different host plants

Host Plant	Endophytes isolated	Reference
Erythrina crista-galli	**Bacteria**: *Arthrobacter citreus, Corynebacterium insidiosum, Enterobacter dissolvens, Pseudomonas fluorescens* **Yeasts**: *Nematospora coryli, Schizosaccharomyces octospora, Sporobolomyces roseus* **Fungi**: *Aspergillus ochraceus Absidia glauca, A. glauca,, Paecilomyces variotii, Penicillium islandicum, Penicillium notatum, Zygorhynchus moelleri*	Weber *et al.*, 2005
Withania somnifera (L.) Dunal	**Ascomycota**: *Chaetomium bostrycodes, Eurotium rubrum, Melanospora fusispora,* **Deuteromycota**: *Aspergillus awamori, Aspergillus auricomus, Aspergillus flavus, Aspergillus niger, Aspergillus pulvinus, Aspergillus terreus, Aspergillus terreus var. aureus, Aspergillus terricola, Aspergillus thomii, Cladosporium cladosporioides, Alternaria alternate, Curvularia oryzae, Drechslera australiensis, Fusarium moniliforme, Fusarium semitectum, Myrothecium roridum, Penicillium corylophilum, Penicillium sp., Phoma sp.*	Khan *et al.*, 2010
Solanum rubrum	**Hypomycetes**: *Aspergillus versicular, Aspergillus fumigatus, Aspergillus niger, Aspergillus sydowi, Aspergillus fonsecaeus, Curvularia lunata, Curvularia geniculate, Penicillium purpurogenum, Penicillium lanosum, Penicillium oxalicum, Trichoderma viridae, Trichoderma lignorum* *Coelomycetes: Colletotrichum sp*	Jena *et al.*, 2013
Morinda pubescence	**Hypomycetes**: *Aspergillus clavatus, Aspergillus fumigatus, Aspergillus versicular, Aspergillus sydowi, Aspergillus flavus, Asperigillus sp., Curvularia lunata, Curvularia interseminata, Curvularia subulata, Penicillium purpurogenum, Penicillium albidum, Trichoderma koningi, Cladosporium herbarum, Nigrospora zimmermann, Nigrospora sphaerica, Torula sp.* **Ascomycetes**: *Chaetomium dolichotrichum, Chaetomium globosum* *Coelomycetes: Colletotrichum sp*	Jena *et al.*, 2013

chemistry, automated synthesis of structrally related small molecules and revolves around certain basic chemical structure besides, screening by machines. Natural products have untold diversity of chemical structures. The chemical diversity of endophytes is unparalleled by even the largest combinatorial database. It requires lot of time to pick, and choose a biological source. Isolation of active natural products, decipher their structure, the natural product may serve as lead molecule whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry. The traditional approaches in medicines include use of natural products. Chinese are the largest user of traditional medicines. They have 5000 plants and plant products in their pharmacopia. Tribal groups in our country also use myriad of plants and plant parts for treating various diseases. 3000 years ago fungi grown on roasted green corn were used to treat intestinal ailments. In 800 AD Papaver sominiferum was used as anestheitc and pain reliever. These products were used without the knowledge of mechanism of action and chemical nature of bioactive compounds. The plant products, in general enhanced the quality of life, reduced pain and suffering and provided relief. Metabolites and /or byproducts from microorganisms/ plants/ animals. World's best known and universelly used medicine Aspirin (Salicylic acid) is derived from Glycoside salicin found in plants like Salix and Populus. World's first billion dollar drug 'Taxol' is a natural product from Taxus sp. Microbes source of bioactive natural products. This idea was conceived after Pasteur discovered that fermentation was caused by living cells. Discovery of Penicillin from Penicillium notatum. This opened the way of discovery and application of microbial metabolites with activity against both plant and human pathogens.

Since the discovery of the world's first billion-dollar anticancer compound - paclitaxel (Taxol) biosynthesized by *Pestalotiopsis microspora* an endophytic fungus of Himalayan yew, interest in studying such endophytes for their medicinal potential has grown tremendously (Strobel, 1996). Natural products (Table 2) from endophytes have a broad spectrum of biological activity and can be grouped into several categories such as alkaloids, steroids, terpenoids, isocumarins, quinones, phenyl propanoids, lignans, aliphatic metabolites, lactones etc. (Zhang *et al.*, 2006). Puri *et al.* (2005) isolated a novel Camptothecin producing endophytic fungus *Entrophosphora inferquens* from an important Indian medicinal plant *Nothapodytes foetida. E. inferquens* synthesizes camptothecin having potential immunomodulatory activity. Similarly, Chen *et al.* (2007) isolated an endophytic fungus *Pencillium thomi* from the roots of *Bruguiera gymnorhiza*. The separation of endophytic fungus from the root led to the isolation of a new compound 4', 5 dihydroxy -2, 3 dimethoxy 4(-hydroxy propyl)- biphenyl along with 11 known compounds. Their effect against three human cell lines was also investigated. Cardiac glycosides Digoxin (C41H64O14) and Digitoxin (C41H64O13) were the main important bioactive compounds extracted from *Digitalis lanata* and *Digitalis purpurea* respectively, were also isolated from their respective endophytes [Ahmed et al, 2012]. Similarly, steroidal saponin, diosgenin (C27H42O3) and a glucoside, namely Aucubin (C13 H19 O8 H2O) were isolated from *Dioscorea bulbifera* from *Plantago ovate* as well as from their endophytes (Ahmed *et al*, 2012).

Endophyte and biodiversity

It is hypothesized that the ecosystems having greatest biodiversity seem to be the ones also having greatest number of endophytes. Tropical and temerate rainforests are the most biologically diverse terrestrial ecosystem on earth. They occupy only 1.44% of land mass but harbor 66 % of world's terrestrial biodiversity. Areasof high plant endemism would possess specific endophytes.Biological diversity implies chemical diversity. Chemical inovation exists in ecosystems where evolutionary race survives. Tropical rain forests are remarkable example of this type of environment. Competition is graet, resources are limited and selection pressure is high. Therefore, there is high probability of novel biologically active compounds i.e. chemical evolution. Host influence general metabolism of endophytic microbes. Reasons of production of identical phytochemicals (host and endophytes), Some say it is because of genetic recombination between the two during evolution, Biology of interrelatedness of endophytes not known, It is not exactly known what an endophyte produces in culture and what it may produce in nature, exact relationship and mode of interaction with plants.

Future research prospects and Conclusion

They are important components of terrestrial ecosystem. They are diverse yet fundamental aspects of their interaction with host is not known. Enormous opportunities exist for the recovery of novel fungal forms, taxa and biotypes. Each plant species harbour at least one endophyte but vast majority of plants have not been studied so for their endophyte associates. More and more plants from unique environmental settings, especially those with unusual biology, and possessing novel strategies of survival should be studied with an objective to unraveal novel chemicals to be utilized for human welfare. Some important points like the processes of endophyte-plant interactions and their relationships with their co-evolutionary patterns and response of host-microbe units to changing environments together or separately need to be deciphered for proper understanding of this exciting relationship. Understanding is also to be developed of genetic bases and phenotypic plasticity of traits of the microbe-plant unit, the use of controlled microbe-plant genotype combinations in different environments. If the endophyte-plant interactions follow similar evolutionary and ecological processes as other host mutualists, host-parasite or host-disease interactions also needs explanations. This relationship helps the host to fight stressed situation and is the store house of diverse chemicals that have multifarious utility which can save the humanity from sufferings. Isolation of endophytic fungi from medicinal and other plants may result in methods to produce biologically active agents for biological utilization on a large commercial scale as they are easily cultured in laboratory and fermentor instead of harvesting plants and affecting the environmental biodiversity this can be used as a strategy for plant conservation.

Table 2: Secondary metabolites produced by different endophytic fungi isolated from different hosts

Sl. No	Host Plant	Endophytic fungi	Secondary metabolites	References
1	*Fragraea bodenii*	*Pestalotiopsis jester*	Quinones	Li et al., 2001
2	*Geotrichum sp.*	*Crassocephalum crepidioides*	Isocoumarin derivatives	Kongsaeree et al., 2003
3	*Artemisia annua*	Unidentified fungus CR115	Diterpenes	Brady et al., 2000
4	*Trachelospermum jasminoides* and *Artemisia annua*	*Myrothecium roridum*	Terpenoid	Shen et al., 2006
5	*Taxus chinensis*	*Gliocladium* sp.	Steroids	Zhang et al., 2002
6	*Cynodon dactylon*	*Aspergillus fumigatus* CY018	Steroids	Liu et al., 2004
7	*Murraya paniculata*	*Eupenicillium* sp	Quinazolines	Barros et al., 2005
8	*Catharanthus roseus* (L.) G. Don	*Fusarium oxysporum*	Indole derivatives	Zhang, et al., 2000
9	*Maytenus hookeri*	*Chaetonium globosum*	Indole derivatives	Zhang, et al., 2002
10	*Imperata cylindrical*	*Chaetosphaeridium globosum* IFB-E019	Indole derivatives	Ding et al., 2006
11	*Terminalia morobensis*	*Pestalotiopsis microspora*	Phenols and phenolic acids	Strobel et al., 2002 and Harper et al., 2003
12	*Erythrina crista-galli*	*Phomopsisi* sp.	Lactones	Weber et al., 2004
13	*Melia azedarach* L	*Aspergillus fumigatus*	Tryprostatin	Zhang et al., 2013

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Antifungal Agents for Treatment of Mycoses

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Being eukaryotes the similarity of fungi with animals in great extent, it is very difficult to develop suitable antifungal compounds which target only to the fungi and spare the host compare to anti-bacterials. Concerted and systematic programmes to discover and develop new antibiotics and anti-fungals have been driven to a considerable extent by the development of resistance by these organisms to the drugs commonly used against them as well as the side effects they exerted on host body. Following are the unique cellular or biochemical targets available in true fungi which could be very effective in designing antifungal drugs:

- Apical tip hyphal growth or by budding.
- Presence of chitin and β-glucans in the cell wall
- Presence of nuclear membrane during cell division.
- Presence of ergosterol in the cell membrane.
- Microtubules are insensitive to colchicines but sensitive to griseofulvin and benzimidazole.
- Lysin biosynthesis by amino adipic acid pathway.
- Stop codon UGA codes tryptophan in fungi

Before discussion about antifungal agents a brief knowledge about different fungal diseases of human beings would help us in understanding the subject. Fungal diseases are usually divided into five groups according to the level of infected tissue and mode of entry into the host which are: superficial, cutaneous, subcutaneous, systemic, and opportunistic infections.

The superficial mycoses occur mainly in the tropics and include black piedra, white piedra, and tinea versicolor. The cutaneous mycoses are which infect the outer layer of skin such as ringworms, tinea, or dermatomycosis. These diseases occur worldwide and represent the most common fungal diseases of humans. Most common such types of mycoses are:

Tinea capitis: Disease of Scalp hair (*Tricophyton* spp. and *Microsporum* spp.)
Tinea corporis: Due to social exchanges and contacts (*Trichophyton* spp.)
Tinea cruris: Disease of joints and groins, itching (*Epidermophyton* sp.)
Tinea pedis: Athletes foot, in bengali 'haza' (*T. rubrum*)
Tinea manuum: similar disease on hands (*T. rubrum*)
Tinea unguium: Attacking nails (*T. rubrum*)

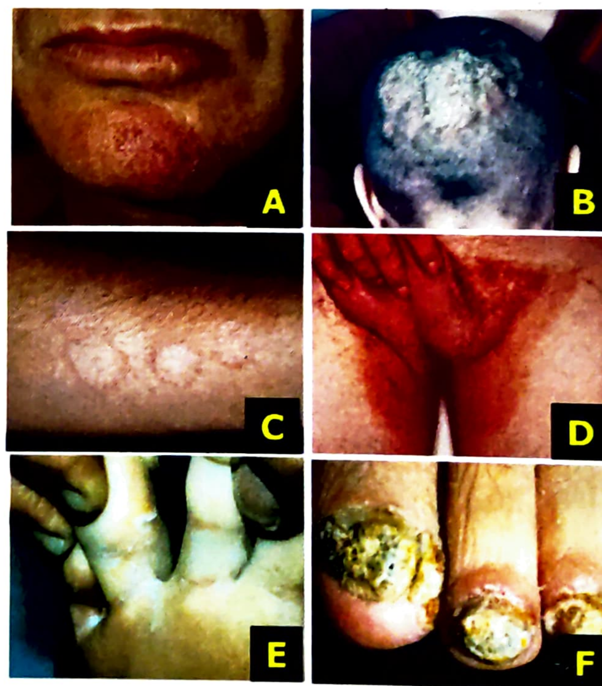

Fig.1. Different types of cutaneous mycoses caused by human pathogenic fungi; A. *Tinea barbae*; B. *Tinea capitis*; C. *Tinea corporis; D. Tinea cruris*; E. *Tinea pedis*; F. *Tinea unguium*

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Fungi that infect below the upper layer of skin are subcutaneous pathogens. Normally they come from soil and must be introduced into the body beneath the cutaneous layer. Examples of such mycoses include chromomycosis, maduromycosis, sporotrichosis. The systemic mycoses are the most serious of the all fungal infections in the normal host because the responsible fungi can disseminate throughout the body. Examples include blastomycosis, coccidiomycosis, cryptococcosis, and histoplasmosis. And lastly, the opportunistic mycoses can create life threatening situations in the compromised hosts. Examples of such diseases include aspergillosis, candidiasis, and *Pneumocystis carinii* pneumonia. Systemic and opportunistic mycoses are prevalent in immunocompromised persons such as AIDS patient or in persons suffering from prolong malnutrion. The systemic pathogens may even invade lung tissues, pericardial membrane to reach heart valves, meninges and spinal cord by a basidiomycetous yeast *Cryptococcus neoformans*, blood stream by *Histoplasma capsulatum* and threaten the lives of patients.

A number of antifungal agents are available today for treatment of mycoses targeting at different sites of cellular and biochemical machineries of fungal cells essential to fungal living. One main difference between the fungal cell and the mammalian cell is the presence of a fungal cell wall, and thus cell wall inhibitors could represent a promising class of antifungal agents. However, only recently have efficient cell wall biosynthesis inhibitors called echinocandins such as caspofungin, micafungin been studied as antifungal agents. These compounds mainly inhibit fungal glucan biosynthesis which makes their cell wall. Beauty of these compounds is the total absence of their cross resistance and minimum toxicity, and they are fungicidal in their mode of action. An antibiotic nikkomycin Z also does the same function by inhibiting chitin biosynthesis.

Membrane lipids including sterols have great role for its stabilization and functioning. Sterols are important constituents of both mammalian and fungal cell membranes, but there is a significant difference that allows fungal cells to be selectively targeted. Mammalian cell membranes contain cholesterol as the predominant sterol component, while fungal cell membranes contain ergosterol as the primary steroid component. These two sterols are quite similar in structure, but this structural difference has become the basis for the activity of many available antifungal agents.

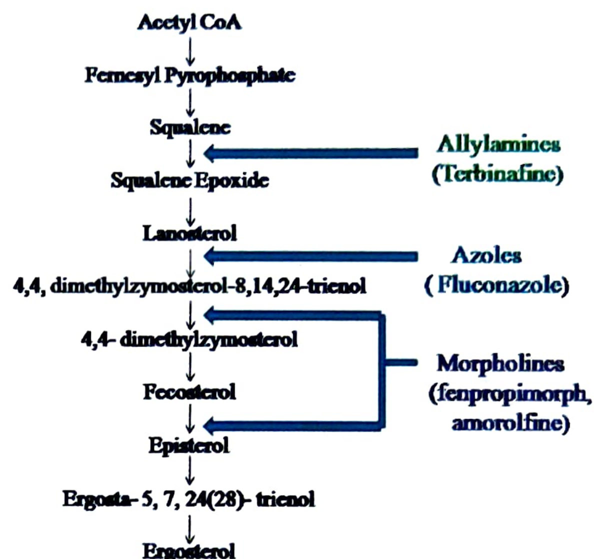

Fig. 2: Inhibition of ergosterol bio-synthesis by different antifungal drugs.

The discovery of polyene antifungal agents can be considered the first significant breakthrough in antifungal therapy. Polyene antibiotics such as nystatin and amphotericin B have an affinity for cell membranes that contain ergosterol rather than cholesterol, and as such are reasonably well targeted to fungal cell membranes. These antibiotics integrate themselves into the cell membrane of fungi, causing the membranes to become leaky, and ultimately to lyse, killing the organism. However, both drugs are quite toxic to the mammalian host, and thus must be used with caution. Nystatin is too toxic to be used systemically. However, it has very poor bioavailability when given orally, and thus it can be used to treat fungal infections of the mouth and GI tract. Amphotericin B has a low enough toxicity to be used systemically by IV administration, but can produce significant nephrotoxicity that limits its use as a systemic antibiotic. Some newer formulations of amphotericin B such as different lipid formulations have been developed with a somewhat attenuated toxicity profile.

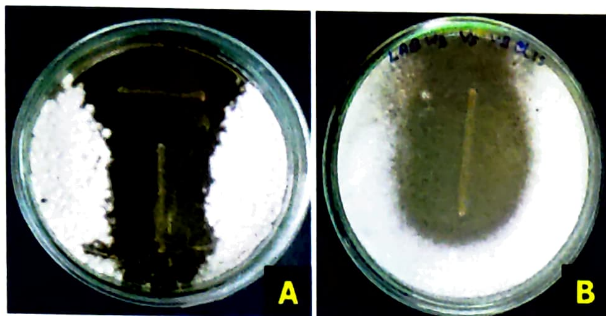

Fig. 3: Zones of inhibition produced by lactic acid bacteria against fungal pathogens: A- *Mucor* sp. B- *Curvularia* sp.

A number of agents have been developed that target the biosynthesis of ergosterol, which is unique to the organism. A key step in the fungal biosynthesis of ergosterol is the cytochrome P450 enzyme 14-·- demethylase (known as CYP51), and many of the available agents target this enzyme as their primary mechanism of action. Treatment with an azole results in the accumulation of sterols still bearing a 14- methyl substituent, and these results in permeability changes, leaky membranes and malfunction of membrane-bound proteins. The first azole based drug marketed was ketokonazole for therapeutic purpose in 1958 although its efficacy was determined a decade ago. Later the second generation azoles like fluconazole and recently with very high therapeutic indexed azoles like voriconazole and posaconazoles are marketed by renouned companies like Pfizer and Schering.

A group of allyl amines and some related derivatives have been developed that inhibit the fungal enzyme squalene synthase, a step in the synthesis of cholesterol and ergosterol. Selectivity for the fungus is based on the fact that fungal squalene synthase is much more sensitive to drug treatment than the mammalian form of the enzyme. The most important drugs in this regard are terbinafine and tolnaftate.

Of the other antifungal agents that can be used to treat fungal infections, sodarins are important. This is a group of compounds inhibit many fungus-specific proteins with moderate precision thus inhibit their growth effectively in fungi, the drug flucytosine is converted to 5-fluorouracil by fungal cytosine deaminase, and then through a series of steps to 5-fluorodeoxyuridine monophosphate. 5- Fluoro-deoxyuridine monophosphate acts as a thymidylate synthase inhibitor in fungi, which interferes with the ability to synthesize RNA and some proteins, resulting in the death of the organism. These transformations do not occur in mammalian cells. However, some bacteria in the human intestinal flora can convert flucytosine to 5-fluorouracil, which is used as a cytotoxic agent in cancer chemotherapy, so human toxicity can result. Resistance to flucytosine is a significant problem, and as such the drug is generally used in combination with amphotericin B.

Fungal microtubules of the spindle are seriously damaged by Griseofulvin, an antibiotic produced by *Penicillium patulum* can and thus stop fungal growth. It can be used orally for the treatment of fungal infections of the fingernails and toenails. Topical griseofulvin does not

Table 1. A list of fungi susceptible to inhibitory compounds produced by lactic acid bacteria

LAB Isolate	Activity Spectrum	Compound(s)	References
Lactobacillus plantarum MiLAB14	Broad antifungal Spectrum	Hydroxy fatty acids, Phenyl lactic acid, Cyclo (Phe-Pro), Cyclo(Phe-OH-Pro)	Sjogren et al., 2003
Lactobacillus rhamnosus	*Penicillium* spp. *Aspergillus* spp. *Fusarium* spp. *Alternaria* spp.	Sodium acetate1	Stiles et al., 2002
Lactobacillus casei	*Penicillium* spp.	Possibly proteinaceous	Gourama, 1997
Pediococcus pentosaceus MiLAB24	Broad Spectrum	Cyclo (Phe-OH-Pro)	Magnusson et al., 2003
Lactobacillus pentosus	*Candida albicans*	Pentocin TV35b	Okkers et al., 1999
Pediococcus acidilactici LAB5	Broad antifungal spectrum	Phenyl lactic acid, An unknown molecule of 83kDa	Mandal et al., 2013

penetrate skin or nails, but when given orally, it is incorporated into keratin precursor cells, and ultimately into the keratin that makes up skin and nail tissue. This form of keratin cannot support fungal growth. The mechanism of action for griseofulvin involves binding to tubulin, which inhibits cell division, and it may also interfere with DNA replication.

Undecylenic acid is widely employed in OTC preparations, and is fungistatic when applied topically, presumably because it interacts with constituents of the fungal cell membrane.

Application of probiotic microorganisms also reduced the food and fungi mediated allergies in human beings. These also maintain the healthy micrbiota of our intestinal system and help us to combat many pathogenic infections particularly *Candida albicans* thus offering a great prospect for future use against fungal diseases. Many species of *Lactobacillus* and some species of *Pediococcus* and *Lactococcus* including *P. acidilactici* LAB5, *L. lactis* sub species *lactis* LABW1 and *L. lactis* sub species *lactis* LABW 3 of our isolates could kill a significant number of human- as well as plant- pathogenic fungi. The *Pediococcus* isolate produces phenyl lactic acid and an unknown compound of 83 KD as the key molecules responsible for antifungal activity.

A number of different mechanisms contribute the development of resistance. They include molecular changes of the drug itself; over expression of drug target-thus swamping the antifungal agent; the reverse over expression, namely reduction in concentration of drug target-thus eliminating it as a site of action; changes in molecular biosynthesis; and pumps that actively eliminate the antifungals. Dissection of each of these mechanisms reveals new weaknesses in the pathogens and is used as a strategy to combat the problem of resistance. Genomic decoding of many of the pathogenic fungi will help accelerate the validation of multiple targets against which new generations of antifungals are beginning to develop.

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EFFICACY OF ANTHOCYANIN IN PRODUCTION OF REMEDIAL TEA

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ABSTRACT

Tea is consumed as a medicinal beverage from centuries, as the medicinal component includes polyphenols, caffeine, and amino acids. Apart from that it also contains flavonoids; compounds reported to have antioxidant properties with many beneficial effects. Anthocyanins belonging to the flavonoid group are naturally occurring compounds that imparts colour to fruits, vegetables and plants. Apart from that it has an array of health promoting benefits. This article has been reviewed to highlight the importance of anthocyanin as well as to motivate research in exploring tea varieties with abundant anthocyanin so that consumption of anthocyanin rich tea or beverage and also use of natural dye made using anthocyanin would replace harmful effects of chemical drugs and also improve country's economy by flourishing the tea industry with increased consumption.

Keywords: *Camellia sinensis*, anthocyanin, pharmacological activity, industrial use

Introduction

Cultivated tea belongs to genus *Camellia*, consisting of three species each with specific plant types viz. *Camellia sinensis* China type, *Camellia assamica* Assam type and *Camellia assamica lasiocalyx* Cambod type (Wight, 1962). Tea is an important agricultural and commercial crop consumed worldwide, mainly as a beverage made from processed tea leaves and it has also been used for medicinal purposes for several centuries (Friedman *et al.*, 2007; Wang *et al.*, 2012). According to varying processing procedures, tea currently made in the world can be classified into six main types including black, green, white, yellow, oolong and reprocessed tea. Of all the types, green tea is mostly favoured as a medicinal tea as many of its medicinal properties like antioxidative, anti-mutagenic, anticarcinogenic, anti hypersensitive, anti bacterial, antiviral and also weight reducing property have been already reported (Saito et al., 2011). Besides green tea, purple tea is gaining much importance since, interest in anthocyanins is growing among researchers owing to their potential health benefits (Kong et al., 2003).

Anthocyanins are the another most important plant pigment besides chlorophyll visible to the human eye belonging to the widespread class of phenolic compounds collectively named flavonoids (Kong *et al.*, 2003). They occur in different colour basically red, blue or purple depending upon their pH. Synthesized through the phenylpropanoid pathway, the water soluble vacuolar pigment has many important roles to play besides imparting colour and contributing to astringent sensation.

It is found in many plant species including red grapes (Rivero-Perez *et al.*, 2008), berries (blueberry, strawberry, raspberry, blackcurrant, bil- berry, cranberry, elderberry) (Nicoue *et al.*, 2007), eggplant (Azuma et al., 2008), purple fleshed sweet potatoes (Oki *et al.*, 2002) and flowers like *Hibiscus* (Lo *et al.*, 2007a). These pigments have been found to be the largest and most important group of water soluble pigments found in nature and they contribute to the attractive colours of fruits, vegetables and flowers imparting red, orange, purple, violet and blue colours (Feild *et al.*, 2001). Anthocyanins in plants normally accumulate in the vacuoles of the epidermal and sub epidermal cells (Steyn *et al.*, 2002). The colours of these pigments are pH dependent (Mazza and Miniati, 1993). Interest in anthocyanins has recently increased owing to their potential health benefits (Kong, Chia, Goh, Chia, & Brouillard, 2003) and their use as an alternative source of synthetic colourants/dyes (Jackman *et al.*, 1987; Kerio *et al.*, 2012).

The anthocyanin pigments that create the

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color (Tsai and Ou, 1996) are responsible for the wide range of coloring in many foods. Recently, the biological activities of anthocyanin, such as antioxidant activity, protection from atherosclerosis and anti-carcinogenic activity have been investigated, and shown to have some beneficial effects in the treatment of diseases.

For instance, there have been reports on the antioxidant activity in grape anthocyanin (Igarashi *et al.,* 1989) and the biological effect of anthocyanin in low density lipoprotein and lecithin-liposome systems (Meyer *et al.,* 1997). Anthocyanins were also found to have many times more activity than common antioxidants such as ascorbate (Wang *et al.,* 1997). Overall, there is now increasing evidence that antioxidants in the human diet are of major benefit for health and well-being. (Tsai *et al.,* 2002)

However, no tea has been demonstrated to contain an abundant amount of anthocyanins. Therefore, 'Sunrouge' was developed, a red leaf tea cultivar that is rich in anthocyanins by natural crossing in 2009. An anthocyanin-rich parental line, 'Cha Chuukanbohon Nou 6', which was derived from *C. taliensis x C. sinensis* in 2004, was previously developed. *C. taliensis* is closely related to *C. sinensis.* However, the anthocyanin content of Cha Chuukanbohon Nou 6'suddenly diminishes as the leaf matures. Therefore, anthocyanin rich tea was developed the cultivar which was higher in anthocyanin content than 'Cha chuukanbohon Nou 6', and in which the anthocyanin content did not diminish after leaf maturation. 'Sunrouge' is an offspring of 'Cha Chuukanbohon Nou 6'. Saito *et al.* isolated six anthocyanins from 'Sunrouge' leaves (Maeda-Yamamoto *et al.,* 2012). Because of the sedentary nature of plants, they are prone to UVirradiation which can cause oxidative stress. Anthocyanins protect plants against such irradiation. Their biosynthesis has been demonstrated to be upregulated when the plant is exposed to UV-B irradiation (Merzlyak *et al.,* 2008). Although anthocyanin is not found abundantly in Camellia sinensis, there are many more purple leaf coloured tea plants that are yet to be explored to make promising anthocyanin rich tea.

Biochemistry of Anthocyanin

Anthocyanin belongs to the widespread class of phenolic compounds collectively named flavonoids. They are glycosides of poly-hydroxy and poly-methoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (Kong *et al.,* 2003). Chemically, anthocyanins are glycoside moieties of anthocyanidins derived from the flavylium (2-phenyl benzopyrilium) cation shown in Fig 1 (Kerio *et al.,* 2012). The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule (Kong

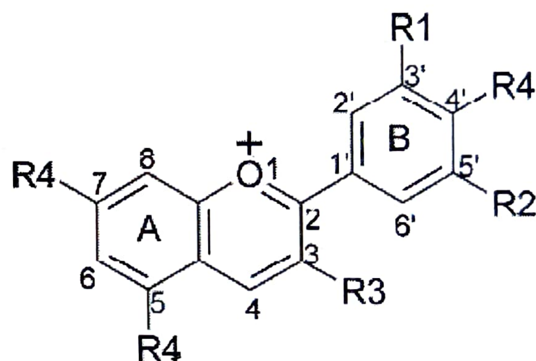

Fig 1: Basic structure of anthocyanidin pigment, the flavylium cation where R1 and R2 are H, OH, or OCH; R3 is a glycosyl or H; and R4 is OH or a glycosyl. *(Kong et al.*, 2003)

There are several anthocyanidins described in nature but among these, six are widespread in fruits and vegetables namely; pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin as shown in Table 1 (Kerio et al., 2012). The various anthocyanidins differs in number and position of the hydroxyl and also methyl ether groups attached on 3, 5, 6, 7, 3', 4' or 5' positions (Table 1). Despite the fact that 31 different monomeric anthocyanidins have been identified (including 3-deoxy anthocyanidins, pyrano-anthocyanidins and sphagnorubins), 90% of the naturally occurring anthocyanins are based on only six structures (30% on cyanidin, 22% on delphinidin,18% on pelargonidin and in summary 20% on peonidin, malvidin and petunidin). Those six anthocyanidins are usually known as common

Table 1: Common anthocyanidins occurring in the nature. Excerpted from Ananga *et al.* (2013)

ANTHOCYANIN COMMON IN HIGHER PLANTS										
Sl. no	Name	Abbreviation	Substitution Pattern							
			3	5	6	7	3′	4′	5′
1	Cyanidin	Cy	OH	OH	H	OH	OH	OH	H
2	Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH
3	Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe
4	Pelargonidin	Pg	OH	OH	H	OH	H	OH	H
5	Peonidin	Pn	OH	OH	H	OH	OMe	OH	H
6	Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH

anthocyanidins (Ananga *et al.,* 2013).The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides. 3-glycosides occur about two and half times more frequently than 3,5-diglycosides. So, the most widespread anthocyanin is cyanidin 3-glucoside. (Kong *et al.,* 2003)

Because of their polar nature, anthocyanins are soluble in polar solvents, such as methanol (MEOH), ethanol and water. The initial step in their isolation therefore involves solvent extraction, which includes the use of acidified methanol or ethanol. The use of acid stabilizes anthocyanins in the flavylium cation form, which is red at low pH (Mc. Ghie and Walton, 2007).

The color of anthocyanidins differs with the number of hydroxyl groups, attached on their molecules (especially those substituted in ring B). With the increase of attached hydroxyl groups, the visible color of entire molecule shift from orange to violet (Andersen and Jordheim, 2006; Delgado-Vargas et al., 2000; Delgado-Vargas and Paredes-Lopez, 2002; Tanaka *et al.,* 2008).

Glycosylation of anthocyanidins results to additional reddening of obtained anthocyanins, whereas the presence of aliphatic or aromatic acyl moieties causes no color change or slight blue shift and has significant effect on their stability and solubility (Tanaka *et al.,* 2008). Changes in pH can also cause reversible structural transformations in anthocyanins molecules, which has a dramatic effect on their color Delgado-Vargas and Paredes-Lopez, 2002; Wrolstad, 2004). Most of the anthocyanins are *O*-glycosylated at 3 (except those based on 3-deoxyanthocyani-dins and sphagnorubinns), 5 or 7 positions and in some cases at 3′, 4′ and 5′ positions (Ananga *et al.,* 2013). However, 8-*C*-glycosylanthocyanins have been found only in *Tricyrtis formosana* Baker (Saito *et al.,* 2003; Tatsuzawa *et al.,* 2004). Anthocyanins contain two, one or tree monosaccharide units in their molecules. The usual monosaccharide residues are glucose, galactose, arabinose, ramnose, xylose and glucuronic acid. However, anthocyanins containing disaccharides and trisaccharides were also found in nature but no tetrasaccharides have been discovered yet (Ananga *et al.,* 2013).

Biosynthesis of Anthocyanin in Tea

Anthocyanin molecules are produced via flavonoid pathway. Anthocyanin pigments are assembled like all other flavonoids from two different streams of chemical raw materials in the cell where one stream involves

the shikimate pathway to produce the amino acid phenylalanine and the other stream produces three molecules of malonyl - CoA, a C3 unit from a C2 unit (acetyl - CoA). The Shikimate pathway leads to the formation of chorismate, which is the precursor of the aromatic amino acids phenylalanine, tyrosine and tryptophan. Phenylalanine (and in some cases tyrosine, but not in the case of tea plants) is the primary precursor of catechins (Tounekti *et al.*, 2013). The flavonoid pathway starts with phenylalanine, produced via shikimate pathway and trans- formed to 4 coumaroyl - CoA. The key enzymes, chalcone synthase (CHS) produce a naringen in chalcone by condensing one molecule of 4-coumaroyl - CoA and three malonyl - CoA molecules (derived from citrate produced by The Krebs cycle). In this case, the rings A and C are derived from the acetate pathway, whereas the ring B is derived from shikimate pathway (Ananga *et al.*, 2013). Currently there are three isoforms of chalcone synthase (Park *et al.*, 2004). The three genes act to synthesize naringenin chalcone, which is used in the formation of anthocyanins, proanthocyanidins, and other phenolic compounds. According to (Ageorges *et al.*, 2006), the three different CHSs may act in three different pathways to produce different secondary metabolites. In the next step, chalcone isomerase (CHI) converts stereospecifically the naringenin chalcone to its isomer naringenin. Ring B of the naringenin undergoes further hydroxylation by the enzymes flavonoid 3´-hydroxylase (F3´H), flavonoid 3´5´-hydroxylase (F3´5´H) or flavanon 3ß-hydroxylase (F3H) (He *et al.*, 2010). Then, the obtained dihydroflavonols are reduced by the enzyme dihydroflavonol 4-reductase (DFR) to the corresponding leucoanthocyanidins. After this reduction, anthocyanidin synthase (ANS) oxidize leucoanthocyanidins to their corresponding anthocyanidins. Anthocyanidins are inherently unstable under physiological conditions and are immediately glycosylated to anthocyanins by UDP-glucose: Anthocianidin: Flavonoid glucosyltransferase (UFGT) (He *et al.*, 2010). Anthocyanins, containing methylated anthocyani-dins (peonidin 4, petunidin 5 and malvidin 6) as aglycone can be obtained by methylation of hydroxyl groups on the ring B of the cyanidin-3-O-glucoside 7, delphinidin-3-O-glucoside and petunidin-3-O-glucoside by the enzyme O-methyltransferase (OMT). Future acylation of produced anthocyanins is possible by the action of different anthocyanin acyltransferases (ACT).

Extraction of Anthocyanin

Anthocyanins are soluble in polar solvents, and they are normally extracted from plant materials by using methanol that contains small amounts of hydrochloric acid or formic acid. The acid lowers the solution's pH value and prevents the degradation of the non-acylated anthocyanin pigments. However, as hydrochloric acid or formic acid is concentrated during the evaporation of the methanol-hydrochloric acid or methanol-formic acid solvent, pigment degradation occurs (e.g. in the extract of Azalea cv. Alice Erauw, the cyanidin-3monosides are converted into unstable aglycone). Small amounts of acid may also cause partial or total hydrolysis of the acyl moieties of acylated anthocyanins that are present in some plants. One report compared various techniques for the extraction of anthocyanins from red grapes and demonstrated that solvents containing up to 0.12 mol/l hydrochloric acid can cause partial hydrolysis of acylated anthocyanins (Revilla *et al.*, 1998). Acetone has also been used to extract anthocyanins from several plant sources (Garcia Viguera *et al.*, 1998; Giusti *et al.*, 1994). In comparison to acidified methanol, this technique allows an efficient and more reproducible extraction, avoids problems with pectins, and permits a much lower temperature for sample concentration (GarciaViguera *et al.*, 1998). Solid-phase extraction (SPE) on C_{18} (SPE) cartridges or Sephadex is commonly used for the initial purification of the crude anthocyanin extracts. The anthocyanins are bound strongly to these adsorbents through their unsubstituted hydroxyl groups and are separated from unrelated compounds by using a series of solvents of increasing polarity. Sunrouge' tea leaves i.e., anthocyanin rich tea leaves (87.2 g) were extracted with 15% acetic acid (600 mL 3), and additionally extracted with 15% acetic acid-containing 50% EtOH (600 mL 4) (Saito *et al.*, 2011). The anthocyanins were extracted

successfully from tea products processed from a number of newly bred purple leaf coloured Kenyan tea cultivars (*Camellia sinensis*) using acidified methanol/HCl (99:1 v/v). (Kerio *et al.,* 2012).

Characterization of Anthocyanin

The characterization of a mixture of anthocyanins usually involves the separation and collection of each compound, and subsequent analysis by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectroscopy (FAB-MS). For the separation and structural analysis, the use of liquid chromatography–mass spectrometry (LC–MS) technique, which combines the separation of LC with the selectivity and sensitivity of the MS detector, permits the identification of individual compounds in a mixture of compounds. Recently liquid chromatography-electron impact ionization mass spectrometry (LC-EI-MS) was also used to identify the anthocyanins of *Catharanthus roseus* extracts (Piovan *et al.,* 1998). LC–MS with an atmospheric pressure-ionization ion-spray interface was used to analyze the anthocyanins contained in the grape skins (*Vitis vinifera* L.). Nineteen derivatives of cyanidin, delphinidin, petunidin, malvidin and peonidin were identified by this ionization technique. The individual mass spectra showed peaks for the molecular ions, together with a fragment corresponding to aglycone; when acylation was present, an additional fragment was detected at mass/charge values corresponding to the loss of acyl moiety from the molecular ion (Baldi *et al.,* 1995). Many new acylated anthocyanins have been found with the help FAB-MS (Saito *et al.,* 1983).

Atmospheric-pressure ionization (API) techniques have several advantages over other MS detection methods. In API-MS the ion source is located outside the MS; the ions are formed at atmospheric pressure, and then sampled into the mass spectrometer. These are soft ionization techniques (only the molecular ion is formed), although the application of a potential at the entrance of the mass spectrometer (fragment voltage) creates suitable conditions for CID, and the production of fragment ions. Two API interfaces are available commercially, namely, the atmospheric pressure chemical ionization interface (APCI) and the ESI interface. LC–MS system equipped with an ESI interface was used to analyze anhocyanins present in extracts of grape skins and red wine (Revilla et al., 1999) Another technique recently used for anthocyanin analysis is capillary electrophoresis (CE) which has excellent mass sensitivity, high resolution, low sample consumption and minimal generation of solvent waste. The separation of a mixture of standards, as well as strawberry and elderberry anthocyanins, by capillary zone electrophoresis (CZE) has already been reported by Bridle et al. (1997). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS) was used to perform both qualitative and quantitative analyses of anthocyanins in wine and fruit juice, and matrix-assisted laser desorption/ionization time-offlight mass spectrometry (MALDI–TOF–MS) to analyze the content of anthocyanins in various foods (Wang and Sporns, 1999; Wang and Lin, 2000). Purification and isolation of 6 anthocyanins from 'Sunrouge' by chromatography, and identification of them by LC/MS/MS and NMR analysis has already been reported (Saito et al., 2011).

NMR, an important technique is useful for structural elucidation of anthocyanin. Some examples of its use are given where it has been reported that the separation of anthocyanins from red radish (*Raphanus sativus*) and their structural elucidation by one- and two-dimensional NMR.

Four anthocyanins were obtained: pelargonidin 3-O- [2-O-(b-glucopyranosyl) -6-O-(trans-p-coumaroyl) -b-glucopyranoside] 5O-(6-O-malonyl-b-glucopyranoside); pelargonidin 3-O [2-O -(b-glucopyranosyl) -6-O- (trans-feruloyl)-b-glucopyranoside] 5-O- (6-O- malonyl-b-glucopyranoside); pelargonidin 3-O-[2-O-(b-glucopyranosyl) -6-O- (trans-pcoumaroyl)-b-d-glucopyranoside] 5-O- (b-glucopyranoside) and pelargonidin 3-O-[2-O-(b-glucopyranosyl)-6O-(trans-feruloyl)-b- gluco-pyranoside] 5-O- (b-glucopyr-anoside). They also investigated the three-dimensional conformation of the molecule by using NOESY techniques, which showed proximity between the hydrogen from the cinnamic acid acylating group and the C-4 of the pelargonidin (Giusti *et al.,* 1998). Similarly,

anthocyanin trisaccharides of *Vaccinium padifolium* were identified by using NMR and other techniques (Cabrita *et al.,* 2000). Anthocyanins from Kenyan teas were purified by C_{18} solid phase extraction (SPE) catridges and characterised by HPLC-UV–Visible. They were identified according to their HPLC retention times, elution order and comparison with authentic standards that were available. Total monomeric anthocyanins were determined by the pH-differential method (Kerio *et al.,* 2012). Anthocyanins content in purple tea was measured by single-pH and the content was much more than that measured by pH-Differential spectrophotometry. The two methods showed good linear relation (R2=0.9927). The effects of interfering substances on quantitative analysis of anthocyanins could be eliminated by using pH-Differential spectrophotometry. The results provided a reliable basis for the measurement of Anthocyanins (Chen and Lu, 2011).

Anthocyanin Rich Tea

Sunrouge is the red tea cultivar made by naturally crossing *Camellia taliensis* and *Camellia sinensis* for which an application for registration was made in 2009. An anthocyanin-rich parental line, 'Cha Chuukanbohon Nou 6', was previously developed by crossing *C. taliensis x C. sinensis* in 2004 where *C. taliensis* is closely related to *C. sinensis*. However, the anthocyanin content of 'Cha Chuukanbohon Nou 6 suddenly diminished after maturation of leaves. Therefore Sunrouge was introduced as an offspring of 'Cha Chuukanbohon Nou 6' whose anthocyanin content did not diminish after leaf maturation (Maeda-Yamamoto *et al.,* 2012). The total anthocyanin content of four tea cultivars: 'Sunrouge', 'Cha Chuukanbohon Nou 6', 'Benibana-cha' (*C. sinensis*), the anthocyanin-rich tea cultivar, and 'Yabukita' (*C. sinensis*), the common tea cultivar in Japan was quantified and it has been already reported that the anthocyanin content of 'Sunrouge' was the highest among 4 tea cultivars, and was 8.4 times higher than that of 'Yabukita'. Purification and isolation of 6 anthocyanins from 'Sunrouge' has already been done using chromatography, and also has been identified using LC/MS/MS and NMR analysis, where the four anthocyanins were identified as delphinidin-3-O- ßD- (6-(E)-pcoumaroyl) galactopyranoside(2), delphinidin-3-O-ßD-(6-(E)-p-coumaroyl)glucopyranoside (3), cyanidin-3-O -ß-D- (6-(E)-pcoumaroyl) galacto-pyranoside (4), and cyanidin-3-O-ßD-(6-(E)-p-coumaroyl)glucopyranoside(5), and the other two were estimated respectively as delphinidin-(Z)-p-coumaroylgalactopyranoside (1), petunidin -(E)-p-coumaroyl galacto pyranoside (6). Compound 3 was found in tea for the first time. In general, anthocyanins has been reported to have various bioactivities, including relieving eyestrain and antioxidative effects, so it is expected that drinking 'Sunrouge' tea would bring in the above bioactivities (Saito *et al.,* 2011).

Another anthocyanin rich tea was extracted from tea products processed from a number of newly bred purple leaf coloured Kenyan tea cultivars (*Camellia sinensis*) using acidified methanol / HCl (99:1 v/v). Extracted anthocyanins were purified by C_{18} solid phase extraction (SPE) catridges and characterised by HPLC-UV–Visible. Of the six most common natural anthocyanidins, five were identified in the purified extracts from purple leaf coloured tea, in both aerated (black) and unaerated (green) teas namely; delphinidin, cyanidin, pelargonidin, peonidin and malvidin. The most predominant anthocyanidin was malvidin in both tea products. In addition, two anthocyanins namely, cyanidin-3-O-galactoside and cyanidin-3-O-glucoside were also identified (Kerio *et al.*, 2012).

Anthocyanin and its Utility in Plants

Apart from imparting colour to the plants, anthocyanin play a definite role in the attraction of animals for pollination and seed dispersal, and hence they are of considerable value in the co-evolution of these plant-animal interactions. Anthocyanins and 3-deoxyanthocyanidins however have roles in flowering plants other than as attractants. They can act as antioxidants, phytoalexins or as antibacterial agents. Anthocyanins may be important factors along with other flavonoids in the resistance of plants to insect attack (Harborne, 1988). For example, cyanidin 3-glucoside was shown to

protect cotton leaves against the tobacco budworm (Hedin and Hedin, 1983). In photosynthetic tissues (such as leaves and sometimes stems), anthocyanins have been shown to act as a "sunscreen", protecting cells from high-light damage by absorbing blue-green and ultraviolet light, thereby protecting the tissues from photo - inhibition, or high-light stress. This has been shown to occur in red juvenile leaves, autumn leaves, and broad-leaf evergreen leaves that turn red during the winter. The red coloration of leaves has been proposed to possibly camouflage leaves from herbivores blind to red wavelengths, or signal unpalatability, since anthocyanin synthesis often coincides with synthesis of unpalatable phenolic compounds (Sullivan, 1998). Some roles of anthocyanin have been tabulated in Table 2.

Pharmacological Activity

Anthocyanins possess known pharmacological properties and are used by humans for therapeutic purposes. Following the recognition that pigment extracts are more effective than O-(b-hydroxyethyl) rutin in decreasing capillary permeability and fragility and in their anti-inflammatory and anti-oedema activitiest is possible that anthocyanins may replace rutin and its derivatives in the treatment of illnesses involving tissue inflammation or capillary fragility. The crude anthocyanin extracts of *Vaccinium myrtillus* have been given orally and by intravenal or intramuscular injection to reduce capillary permeability and fragility. (Kong *et al.,* 2003)

Anthocyanins were not found effective in suppressing tumor growth (Ghiselli *et al.,* 1998). However, an antioxidant activity study of anthocyanin fractions from Italian red wine showed that the anthocyanin fraction was the most effective both in scavenging reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation (Ghiselli *et al.,* 1998). This result suggests that anthocyanins could be the key component in red wine that protects against cardiovascular disease. The anti-tumor activity of anthocyanins was reported where they found that the anthocyanin fraction from red wine suppressed the growth of HCT-15 cells, which are derived from human colon cancer or AGS cells from human gastric cancer. The suppression rate by the anthocyanin fraction was significantly higher than that of the other fractions (Kamei *et al.,* 1998). The ability of anthocyanin obtained from the petals of *H. rosa-sinensis* was examined which prevented carbon tetrachloride-induced acute liver damage in rats. The results showed that those rats treated with anthocyanin and

Table 2: The role of anthocyanins and 3-deoxyanthocyanidins in plants. Excerpted from Kong *et al.* (2003)

Plant	Compound	Origin	Function
	Angiosperms		
Senecio cruentus	Cinerarin	Petals	Pollination
Sorghum	Apigeninidin	Leaf sheath	Phytoalexin anti-microbial antioxidants
	Gymnosperms		
Abies concolor	Petunidin-3- glucoside Cyanidin-3- glucoside	Cone	-
Pinus contorta	Anthocyanin	Leaves	Cold tolerance
Pinus banksiana	-	Seedlings	Photoinhibition tolerance
	Ferns		
Davallia divaricata	Pelargonidin-3-p-coumaryl-glc-5-glc (monardein)	Young leaves	-
Ferns species	Apigenidin	Leaves	-
	Mosses		
Bryum, Splachunm	Luteolinidin-5-glc	Leaves	-
	Liverwort		
Cephaloziella exilifolia	Anthocyanin like	Thallus	-

carbon tetrachloride had significantly less hepatotoxicity ($P<0.05$) than those given carbon tetrachloride alone. This was assessed by measuring the levels of serum aspartate and alanine aminotransferase activities 18 hours after carbon tetrachloride was given. This result suggested that anthocyanin may be protective against carbon tetrachloride-induced liver injury (Obi *et al.,* 1998).

On examining antimutagenicity of water extracts prepared from the storage roots of four varieties of sweet potato with different flesh colors, using Salmonella typhimurium TA 98. Two anthocyanin pigments purified from the purple colored sweet potato 3-(6,6´-caffeyl ferulylsophoroside) 5-glucoside of cyanidin (YGM-3) and peonidin (YGM6), effectively inhibited the reverse mutation induced by heterocyclic amines-mutagen, Trp-P-1, Trp-P-2, and IQ in the presence of rat liver microsomal activation systems (Yoshimoto *et al.,* 1999).

It has been reported that the the administration of anthocyanin dyes from *Aronia melanocarpa* to rats before the intraperitoneal injections of PlateletActivating Factor (PAF) and ceruleine had a beneficial effect on the development of acute experimental pancreatitis in rats (Jankowski *et al.,* 2000). It was revealed that this was due to the reduction of pancreatic swelling and a decrease in lipid peroxidation and adenosine deaminase activity.

They also examined the effect of anthocyanins from Cabernet red wine on the course and intensity of symptoms of experimental diabetes in rats (Jankowski *et al.,* 1999). The results showed that a simultaneous daily administration of anthocyanins obtained from Cabernet red wine and streptozotocin substantially decreased sugar concentrations in the urine and blood serum. These anthocyanins also inhibited the loss of body mass caused by the injection of streptozotocin. Simultaneously, the anthocyanin pigment prevented the generation of free oxygen radicals, and decreased the peroxidation of lipids. The influence of anthocyanins was determined from chokeberries on the generation of autoantibodies to oxidize low density lipoproteins (oLAB) in pregnancies complicated by intrauterine growth retardation (IUGR). An experiment was conducted with a study group of 105 pregnant women (on the turn of trimester two according to LMP) with IUGR (sonographic examination results below the 5th percentile for real gestational age) who were randomly divided into 2 groups. Fifty women were administered anthocyanins and 55 women were given a placebo. There was a control group of 60 healthy pregnant women. They then examined the level of oxidative stress measured by the serum concentration of autoantibodies required to oxidize low density lipoproteins (oLAB). In the anthocyanin group, the oLAB titres decreased from 1104_41 mU/ml before treatment to 752_36 mU/ml in the first month and 726_35 mU/ml in the second month, at $P<0.01$. In the placebo group, the oLAB titres showed a slightly increasing trend: 1089_37 mU/ml before treatment, 1092_42 mU/ml in the first month and 1115_43 mU/ml in the second month, at $P>0.05$. The oLAB titres in the control group were 601_49 mU/ml before treatment, 606_45 mU/ml in the first month, and 614_43 mU/ml in the second month, at $P>0.05$. The results indicated that natural antioxidants (anthocyanins) can be useful in controlling oxidative stress during pregnancies complicated by IUGR (Pawlowicz *et al.,* 2000).

Hibiscus anthocyanins (HAs), a group of natural pigments occurring in the dried flowers of *Hibiscus sabdariffa* L., are used in soft drinks and herbal medicines. Their antioxidant bioactivity has been studied and it appears that HAs can significantly decrease the leakage of lactate dehydrogenase and the formation of malondialdehyde induced by a treatment of tert-butyl hydroperoxide (t-BHP). The *in vivo* investigation showed that the oral pretreatment of HAs before a single dose of t-BHP significantly lowered the serum levels of hepatic enzyme markers (alanine and aspartate amino transferase) and reduced oxidative liver damage. The histopathological evaluation of the liver revealed that *Hibiscus* pigments reduce the incidence of liver lesions including inflammation, leucocyte infiltration, and necrosis induced by t-BHP in rats (Wang *et al.,* 2000).

Their pharmaceutical value has been additionally increased due to their high bioavailability. However, the administration and metabolism of Anthocyanins *in vivo* have been investigated in details mostly in rats, whereas

the detailed studies on humans still are scantly presented in scientific literature (He and Giusti, 2010; Yue *et al.,* 2011).

The colorful anthocyanins are the most recognized, visible members of the bioflavonoid phytochemicals. The free-radical scavenging and antioxidant capacities of anthocyanin pigments are the most highly publicized of the modus operandi used by these pigments to intervene with human therapeutic targets, but, in fact, research clearly suggests that other mechanisms of action are also responsible for observed health benefits (Lila, 2004). Anthocyanin isolates and anthocyanin-rich mixtures of bioflavonoids may provide protection from DNA cleavage, estrogenic activity (altering development of hormone dependent disease symptoms), enzyme inhibition, boosting production of cytokines (thus regulating immune responses), anti-inflammatory activity, lipid peroxidation, decreasing capillary permeability and fragility, and membrane strengthening (Lila, 2004).

The roles of anthocyanin pigments as medicinal agents have been well-accepted dogma in folk medicine throughout the world, and, in fact, these pigments are linked to an amazingly broad-based range of health benefits. For example, anthocyanins from *Hibiscus* sp. have historically been used in remedies for liver disfunction and hypertension; and bilberry (*Vaccinium*) anthocyanins have an anecdotal history of use for vision disorders, microbial infections, diarrhea, and diverse other health disorders (Rice-Evans and Packer, 2003; Smith *et al.,* 2000; Wang *et al.,* 2000).

But while the use of anthocyanins for therapeutic purposes has long been supported by both anecdotal and epidemiological evidence, it is only in recent years that some of the specific, measurable pharmacological properties of isolated anthocyanin pigments have been conclusively verified by rigorously controlled in vitro, in vivo, or clinical research trials (Tsuda *et al.,* 2003). For example, visual acuity can be markedly improved through administration of anthocyanin pigments to animal and human subjects, and the role of these pigments in enhancing night vision or overall vision has been particularly well documented (Matsumoto *et al.,* 2001). Oral intake of anthocyanosides from black currants resulted in significantly improved night vision adaptation in human subjects (Nakaishi *et al.,* 2000) and similar benefits were gained after administration of anthocyanins from bilberries (Muth *et al.,* 2000). Three anthocyanins from black currant stimulated regeneration of rhodopsin (a G-protein-coupled receptor localized in the retina of the eye), and formation of a regeneration intermediate was accelerated by cyanidin 3-rutinoside (Matsumoto *et al.,* 2003). These studies strongly suggest that enhancement of rhodopsin regeneration is at least one mechanism by which anthocyanins enhance visual acuity. In both in vitro and in vivo research trials, anthocyanins have demonstrated marked ability to reduce cancer cell proliferation and to inhibit tumor formation (Lila, 2004). Anthocyanins inhibit tumorigenesis by blocking activation of a mitogen-activated protein kinase pathway. This report provided the first indication of a molecular basis for why anthocyanins demonstrate anticarcinogenic properties.

The role of anthocyanins in cardiovascular disease protection is strongly linked to oxidative stress protection. Since endothelial dysfunction is involved in initiation and development of vascular disease, four anthocyanins isolated from elderberries were incorporated into the plasma. Crude anthocyanin extracts from bilberry have been administered both orally and via injection to reduce capillary permeability (Kong *et al.,* 2003). Protection from heart attacks through administration of grape juice or wine was strongly tied to the ability of the anthocyaninrich products to reduce inflammation and enhance capillary strength and permeability, and to inhibit platelet formation and enhance nitric oxide (NO) release (Folts, 1998).

Their important function in cognitive decline and neural dysfunction has been investigated and found that fruit extracts (from blueberry) including anthocyanins were effective in reversing age related deficits in several neural and behavioral parameters, e.g. oxotremorine enhancement of a K1 evoked release of dopamine from striatal slices, carbachol-stimulated GTPase activity, striatal Ca buffering in striatal synaptosomes, motor behavioral performance on the rod walking and accelerod tasks, and Morris water maze performance and thus proved to improve neural and behavioral

parameters (memory and motor functions). (Joseph *et al.,* 1999).

It has already been reported that the Anthocyanins extracted from purple corn, when provided to mice in tandem with a high-fat diet, effectively inhibited both body weight and adipose tissue increases. Typical symptoms of hyper-glycemia, hyper-insulinemia and hyper-leptinemia provoked by a high-fat diet did not occur when mice also ingested isolated anthocyanins. The experiments suggest that anthocyanins, as a functional food component, can aid in the prevention of obesity and diabetes (Tsuda *et al.,* 2003).

Medicinal Benefits of Anthocyanin Rich Foods

Consumption of anthocyanin adds as a beneficiary source of neutraceuticals and therapeutics and it has various application in the pharmaceutical industry. Recurrent consumption of anthocyanins could provide various health benefits including reduced risk of coronary heart diseases, anti-carcinogenic activity, antioxidant activity, reduced risk of stroke, anti-inflammatory effects etc. (Davies, 2009; Lila, 2004; Stintzing and Carle, 2004; Wrolstad, 2004).

Biological activity of anthocyanin has already been discussed in the above section. Anthocyanin in diet inhibits body weight and adipose tissue increase that could prevent symptoms of hyperglycemia, hyperinsulinemia, and hyperleptinemia provoked by a high-fat diet and thus can aid in the prevention of diabetes and obesity (Tsuda *et al.,* 2003). It improves and cures vision disorders, microbial infections, diarrhea and diverse other health disorders (Rice-Evans and Packer, 2003; Smith *et al.,* 2000; Wang *et al.,* 2000). It has the capacity to modulate cognitive and motor function, to enhance memory, and to have a role in preventing age-related declines in neural function (Lila 2004). It also reduces inflammation and capillary fragility (Kong *et al.,* 2003), scavenges reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation (Ghiselli *et al.,* 1998), provides protection from DNA cleavage, estrogenic activity (altering development of hormone-dependent disease symptoms), enzyme inhibition, boosting production of cytokines (thus regulating immune responses).

Above all anthocyanin has many other health beneficial properties, which include antioxidant (Bae and Suh, 2007), anticarcinogenic (Lee *et al.,* 2009), anti-angiogenic (Bagchi *et al.,* 2004), antimicrobial (Viskelis *et al.,* 2009) antiapoptotic (Elisia and Kitts, 2008) and pro-apoptotic (Lo *et al.,* 2007b) properties.

Utility in Various Industries

The world market of natural food colorants expands with the annual growth rate of 4-6% (Cormier *et al.,* 1996). In USA 4 of the 26 colorants approved by the food administration, that are exempt from certification, are based on anthocyanin pigments (Wrolstad, 2004). In European Union, all anthocyanin-containing colorants are classified as natural colorants under the classification E163 (Socaciu, 2007).

Currently most of the worldwide anthocyanins supply comes from processing of grape pomace, which is a waste product from wine making. But in European Union other plant sources such as red cabbage, elderberry, black currant, purple carrot, sweet potato, and red radish are also allowed (Mortensen, 2006). Anthocyanins, produced by grape cell suspensions can be a promising alternative supply of natural colorants. It has already been demonstrated that the produced pigments by the grape cell suspensions undergo significant structural modifications. Grape cell suspensions accumulates higher levels of metabolically more evolved structures (methylated and acylated anthocyanins). Acylated anthocyanins are suitable for application in food products, mainly because of the improved color stability compared to non-acylated structures (B¹kowska -Barczak, 2005). Moreover, the grape cell suspensions can also produce elevated levels of beneficial phenolic compounds such as flavonoids, stilbenes, phenolics, etc., which are capable of increasing the added value of the final additive. The overall metabolite profile of grape cells in combination with the lack of microbial and toxic contaminations will give the potential for development of new types of food

additives if the entire cell suspension biomass is utilized.

The commercial interest of cosmetic companies to apply plant additives, derived by biotechno-logical cultivation of plant cells to their products has increased remarkably in the last few years (Schurch *et al.,* 2008). The addition of plant cell derived extracts in cosmetic products has been considered as a powerful approach used to increase their health benefits. Several plant extracts have been added to various cosmetic products as moisturizers, antioxidants, whitening agents, colorants, sunscreens, preservatives. With the advancement of plant cell biotechnology, more and more cosmetic companies have been attracted for application of additives, based on plant cell suspensions. Recently the application of so-called plant "steam" cells attracts industry's attention (Schurch *et al.,* 2008). In the last few years, the French company "Sederma" launched the product "Re-sistem™" based on application of *in vitro* cultivated plant cells (www.sederma.fr). The other company, "Mibelle Biochemistry", situated in Switzerland, developed a "PhytoCellTec" prod- uct, based on grape cell suspension of *V. vinifera* L. *cv.* Gamay Fréaux, which was processed by high-pressure homogenizer to produce liposomes for application in cream products (www.mi-bellebiochemistry.com). According to the company, the grape cell derived liposomes contained higher amounts of anthocyanins and when applied on skins serve as strong UV protectors and fight photoaging. The presented examples clearly demonstrate the commercial interest to application of grape cell suspension derived products. However, it is a matter of time for the scientists to develop the biotechnological approach of producing anthocyanins by grape cell suspensions from the frame of experimental scale to commercially applicable products (Ananga *et al.,* 2013).

Anthocyanins have also been employed to produce juices and red wine whose natural colour as well as high antioxidant property adds to the quality of product.

Anthocyanins can also be used as pH indicators because their color changes with pH; they are pink in acidic solutions ($pH < 7$), purple in neutral solutions ($pH \sim 7$), greenish-yellow in alkaline solutions ($pH > 7$), and colourless in very alkaline solutions, where the pigment is completely reduced (Michaelis *et al.,* 1936) and thus, it is employed in many chemical or pharmaceutical industry as well as in the fied of research.

Nowadays anthocyanins are being used widely in organic solar cells because of their ability to convert light energy into electrical energy (Cherepy *et al.,* 1997). The many benefits of using dye sensitized solar cells instead of traditional pn junction silicon cells include lower purity requirements and abundance of component materials, such as titania, as well as the fact they can be produced on flexible substrates, making them amenable to roll-to-roll printing processes (Gratzel, 2003).

Conclusion and Future Prospects

Anthocyanins represent a class of important antioxidants, as they are so common in human foods. In recent years, many papers have been published on the *in vitro* antioxidant activity of anthocyanins and their other functions. However, there are still fewer studies on anthocyanin compared to the studies of other flavonoids. On the other hand tea is a pleasant, popular, socially accepted, economical and safe drink that is initially taken as medicine and later as beverage and now, it has proven well as future potential of becoming an important industrial and pharmaceutical raw material. As green tea, the purple coloured anthocyanin rich tea may also be a popular health drink since anthocyanins has many medicinal properties and is particularly known to be beneficial against cardiovascular diseases, for providing anticancer benefits, improving vision, cholesterol and blood sugar metabolism as discussed above in the article. Most importantly it sports much lower caffeine content than black or green tea which is beneficial in beverage. Anthocyanin content in tea in addition to other polyphenols and other medicinal compounds would add a splendid color as well as an enigmatic healing property.

Based on these facts, this review is directed to highlight the importance of anthocyanins in order to improve further research in this field, discovering tea cultivars or wild tea plant, rich in anthocyanin.

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Bacterial Spot (*Xanthomonas cucurbitae)* of cucurbits: A review

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The Cucurbitaceae also known as cucurbits, are a plant family, sometimes called the ground family, consists of around a hundred genera most of which are edible (Angiosperm Phylogeny Group, 2009). The plants in this family are grown around the tropics and in temperate areas for their edible fruits. These were among the earliest cultivated plants both in the Old and New Worlds. The Cucurbitaceae family ranks among the highest of plant families for number and percentage of species used as human food (Lira and Montes Hernández, 1994). Cucumber, pumpkin, different types of gourds, melons, squash and zucchini are among the important edible plants of the family. The cultivation of these cucurbits is hampered by the attack of many pathogens and insects, out of which *Xanthomonas cucurbitae* (Bryan) Dowson (Syn.: Xanthomonas campestris pv. cucurbitae) causing bacterial spot is emerging as an important pathogen leading to huge crop losses especially to pumpkin, winter squash and bottle gourd (Jarial *et al.,* 2011 and Babadoost, 2012).

Occurrence

This disease was first reported as bacterial leaf spot on Hubbard squash in New York in 1926 by Bryan (Babadoost, 2012). Since then, the disease has been reported to occur on various cucurbits (Gorlenka, 1979, Vlasov, 2005) like squash (Robbs *et al.,* 1972; Alippi, 1989, Kushina *et al.,* 1994), cucumber (Vincent – Sealy, 1978; Marigoni *et al.,* 1988; Sinha, 1989), pumpkin (Pruvost *et al.*, 2008; Lamichhane et al., 2010; Babadoost and Ravanlou, 2012; Salamanca, 2014; Trueman *et al.,* 2014), watermelon (Pruvost *et al.,* 2009; Dutta *et al.,* 2013) and bottle gourd (Jarial *et al.,* 2011) from different countries of world.

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In India, the disease was first reported on cucumber from Bihar in 1989 by Sinha (Sinha, 1989). After that there had been no reports of the disease on any of the cucurbits from India until Jarial *et al* (2011) reported the severe outcome of the disease on bottle gourd in Himachal Pradesh. In addition, the pathogen has been reported to be pathogenic on cucumber, pumpkin and summer squash (Jarial *et al.,* 2011).

Losses

The disease has been reported to cause significant losses in different cucurbits. According to Larazev (2009) yield losses may reach more than 20% in highly susceptible cultivars, and the disease severity sometimes reaches 50-60% at fruit storage in different cucurbits. Babadoost (2012) has reported yield losses varying between 3 to 90 per cent in case of pumpkin fields due to this disease in Illinois. However, the yield losses due to bacterial spot may reach up to 50 per cent in different cucurbits (Anonymous, 2012). In case of bottle gourd, yield losses between 10 to 70 per cent have been reported in Himachal Pradesh, India by Jarial et al (2011). Losses up to 90 per cent have been reported in case of pumpkin by Salamanca (2014). From Canada, up to 60 per cent fruit loss has been reported due to bacterial spot in pumpkin (Trueman *et al.,* 2014).

Symptoms

The symptoms of the disease have been described in details on different crops by various workers. In case of bottle gourd, the symptoms appear on almost all plant parts (Jarial *et al.,* 2011). Initially, the symptoms develop on leaves of any age group as small marginal chlorotic spots, which increase in size towards the centre of the leaf. Later, the necrotic areas develop in

the chlorotic zone which increases in size, merge with each other and occupy almost complete leaf lamina, forming sites with dead brown tissue (Fig 1A). In majority of the cases the necrotic lesions penetrate deeply into the leaf lamina limited by veins, but the necrotic lesions do not drop as in case of angular leaf spot. With the passage of time, the symptoms also develop on stem / vine, tendrils and floral parts as water soaked areas which ultimately turn into necrotic spots (Fig 1B-F). In severe conditions, amber coloured ooze comes out of the vines and this ooze is also visible when the vine is split into two pieces. Finally, the vine completely turns necrotic and dies. In case of attack on female flowers, a rot is observed on the stigma (Fig 1B) and ovary of the flower resulting in no fruit formation.

On fruits, the symptoms are visible on all the age groups. The symptoms on young fruits appear as water soaked spots which develop into a rot ultimately leading to complete rotting of the fruit. On mature fruits, the symptoms develop as small faded spots on fruit peel which later crack and amber coloured ooze appears on the spots. In severe cases, the fruits get deformed and covered with cracks which finally rot (Fig 1G).

In case of pumpkin the symptoms have been described in details by Babadoost (2012). The symptoms on pumpkin leaves appear as small (1-2 mm) and dark lesions, with indefinite yellow margin (Figure 2C). The lesions may coalesce to form larger necrotic areas, usually on leaf margins (Figure 2D). Different types of lesions have been characterized on leaves, which vary in colour and size (Figure 2A-C).

On fruits of pumpkin (Fig 3), the appearance and size of lesions may vary depending upon the rind maturity and the presence of moisture. Initial lesions are small, slightly sunken, circular spots, 1/16 to 1/4 inch in diameter, with a beige center and a dark-brown halo. Later, the cuticle and epidermis crack, and the lesions enlarge, reaching up to 1/2 inch in diameter. The large lesions may have scab-like appearance and give rise to tan, raised blisters. On mature fruit, saprophytic fungi often colonize the dead, tan tissue at the center of the lesion. Penetration of the bacteria into the flesh can lead to significant fruit rot in the field or later in storage.

According to Goldberg (2012) symptoms of bacterial spot in pumpkin may appear on both the foliage and the fruit. On the foliage, the disease causes small somewhat round water-

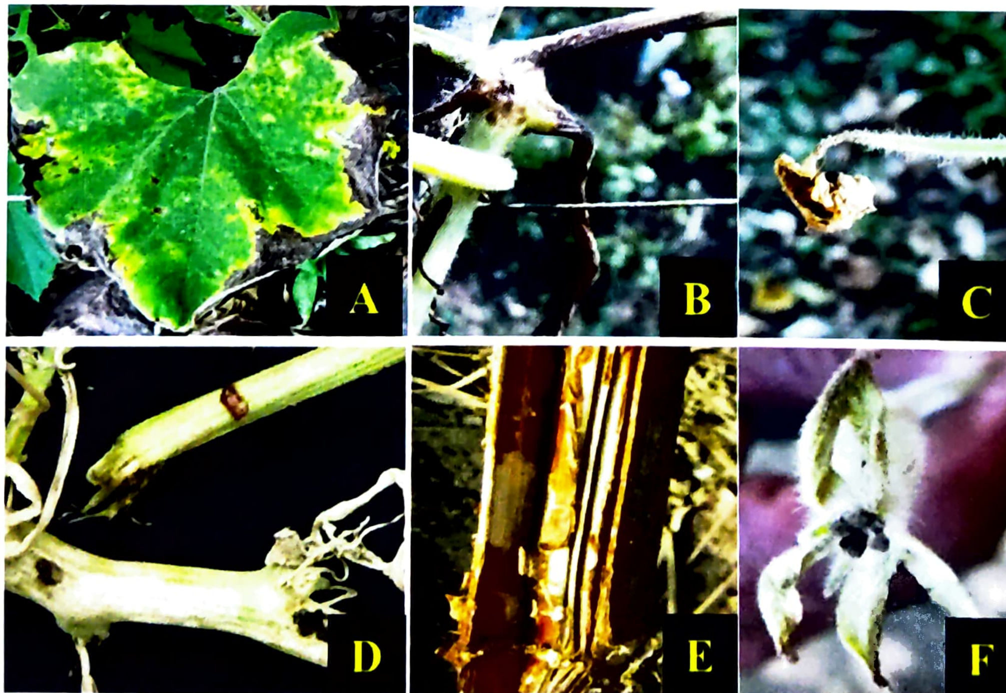

Fig. 1: Symptoms of bacterial spot in bottle gourd (A) in leaves, (B-F) stem, vine, tendril and floral part

soaked lesions on the underside of the leaf. A yellow spot appears on the upper leaf surface. In a few days, the spots turn brown with a distinct yellow halo. Leaf lesions may stay small or enlarge to over 7 mm in diameter. As lesions enlarge, they eventually become delineated by veins resulting in angular lesions. The appearance on fruit is variable and depends on rind maturity and how much moisture is present. Initial lesions are typically small, slightly sunken, mostly round spots with a tan to beige centre. As the spots enlarge (reaching up to 15 mm in diameter), they become noticeably sunken and the rind may crack. Infection extends into the seed cavity of the fruit. The flesh rots and seeds may become contaminated with the bacterium.

In case of water melon angular, water-soaked leaf spots appear which sometimes become necrotic and have a chlorotic halo. Scab-like lesions on fruit can also be observed (Pruvost *et al,* 2009).

According to Salamanca (2014), lesions of bacterial spot appear first on the underside of the leaves as small, water soaked dots that look yellow from the upper side of the leaf. Lesions are especially small (0.07 inches) in pumpkin, winter squash and gourd leaves. As lesions enlarge (0.07-0.15 inches), they can coalesce and look like angular leaf spot. Fruit lesions start as sunken, circular spots (0.04-0.1 inches) that enlarge and can reach up to 0.6 inches in diameter. These openings allow the colonization of the fruit by saprobes or secondary microorganisms that can cause fruit to rot in the field or post-harvest.

Fig 1G: Symptoms of bacterial spot on young and mature fruits of bottle gourd

Causal Organism

The disease is caused by *Xanthomonas cucurbitae* (ex. Bryan) Vauterin *et al.,* 1995, Synonyms *Bacterium cucurbitae* Bryan, *Phytomonas cucurbitae* (Bryan) Bergey et al., *Pseudomonas cucurbitae* (Bryan) Stapp, *Xanthomonas cucurbitae* (Bryan) Dowson, *Xanthomonas campestris pv. cucurbitae* (Bryan) Dye (Lazarev, 2009). The bacterium is gram-negative, oxidase-negative, indole-negative, hydrolyzes starch and esculin, and forms pits on crystal violet pectate and carboxymethyl cellulose media (Dutta *et al.,* 2013). The bacteria can be identified on the basis of morphological, cultural and biochemical characters (Sinha, 1989 and Alippi, 1989). Morphologically, the cells of bacteria are rod shaped and on nutrient agar medium the colonies are mucoid, circular, smooth textured, convex and glistening with entire margins and yellow in colour having diameter of about 3-4 mm. Biochemically, the bacterium hydrolyses asculin as indicated by blackening of asculin medium within 4 days of inoculation, digests protein as evidenced by appearance of clear solution within 10 days of inoculation and liquefies gelatin as observed by appearance of a clear zone around bacterial growth. The

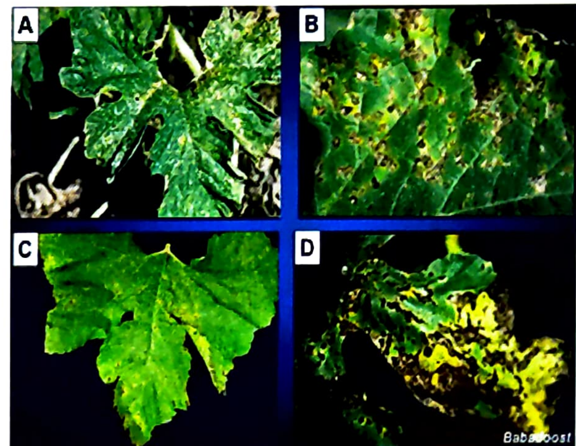

Fig 2: Symptoms of bacterial spot in pumpkin leaves (Courtesy: Babadoost 2012)

bacterium exhibits negative reaction to Gram's stain (Jarial *et al.,* 2011).

Pathogenicity and Host Range

The bacterium has been found to incite disease in almost all cucurbits including cucumber, water melon, pumpkin, prince melon, squash and bottle gourd (Robbs *et al.,* 1972; Taketani *et al.,* 1976; Takikawa and Tsuyumu, 1987; Sinha, 1989; El Hendawy, 1999; Pruvost *et al.,* 2008; and Babadoost and Zitter, 2009; Jarial *et al.,* 2011; Babadoost *et al.,* 2012). According to Babadoost (2013) symptoms were reproduced and pathogen reisolated by artifiacially inoculating various cucrbits including 'Acorn' squash (*Cucurbita pepo*), 'burcucumber' (*Sicyos angulatus*), 'Butternut' squash (*Cucurbita moschata*), cantaloupe (*Cucumis melo*), carving pumpkin (*Cucurbita maxima*), cucumber (*Cucumis sativus*), gourd (*Lagenaria siceraria*), honeydew (*Cucumis melo*), muskmelon (*Cucumis melo*), pumpkin (*Cucurbita pepo*), squash (*Cucurbita maxima*) squash (*Cucurbita argyrosperma*), watermelon (*Citrullus lanatus*), and zucchini (*Cucurbita pepo*). Till date, the host range of the pathogen has been reported to be confined to family cucurbitaceae only (Babadoost, 2013). A bacterial suspension having concentration of 10^5 to 10^8 cfu/ml has been reported to induce pathogenic reaction in different cucurbit hosts by various workers (Pruvost *et al.,* 2009; Jarial *et al.,* 2011; Babadoost and Ravanlou, 2012; Dutta *et al.* 2013; Trueman *et al.,* 2014). An incubation period of 3 to 5 days on inoculated leaves and 5 to 8 days on inoculated fruits of bottle gourd, cucumber, pumpkin and squash plants has been reported by Jarial *et al* (2011) while *Trueman et al* (2014) have reported an incubation period of 10 days in inoculated leaves of pumpkin plants. The symptom development has been reported to be delayed by two days on fruits as compared to leaves (Jarial *et al.,* 2011).

Survival, Disease cycle and Epidemiology

The bacterium is internally seed borne (Vincent—Sealy and Brathwaite, 1982) but the exact location of the bacterium in the seed is not well documented yet (Babadoost, 2013). The disease is seed-borne and may survive on infected crop debris, but is not known to survive in soil (Goldberg, 2012). The bacterium has also been reported to survive in soil only in association with infected leaves and fruits up to 24 months. (Thapa, 2014). The disease is favored by warm temperatures and high humidity or wet conditions caused by frequent rains or overhead irrigation. The disease is

Fig 3: Symptoms of bacterial spot in pumpkin fruits (courtesy: Babadoost, 2012)

spread from plant to plant by rain or irrigation splash, and by movement of people or equipment through the field (Goldberg, 2012). Fruit infection occurs through natural opening or wound in young, rapidly expanding fruit prior to the development of thick, waxy cuticle. The bacteria are splash-spread in the field. Spread of the bacteria within fields can be very rapid. Long distance dispersal of the pathogen is believed to be by contaminated seed (Anonymous, 2012). High temperature (25-30°C) and relative air humidity (90% or higher) are favourable for the disease development. The intensity of the disease incidence increases during the vegetation period and reaches peak at the end of July -and the beginning of August (Lazarev, 2009).

Host Resistance

Much work has not been done on this aspect of the disease. However, Sinha (1989) has reported cv. Japanese Long Green and Collection 72-10 of cucumber to be moderately resistant towards the disease out of 15 genotypes screened. According to Babadoost

(2013), no cucurbit cultivar resistant to *X. cucurbitae* has been determined yet. Similar observations have been documented by Jarial *et al* (2015) in case of bottle gourd where six different genotypes / varieties were observed for disease development and all were found to exhibit susceptible reaction towards the disease.

Management

The disease management strategy should include integration of all effective methods in the area (Babadoost and Zitter, 2009). Various workers in the disease affected regions of the world have mentioned different methods for disease management including cultural practices, seed treatments or foliar sprays. Crop rotation with non-cucurbitaceous crops for two years or longer will reduce the disease levels (Anonymous, 2012; Goldberg, 2012; Babadoost, 2013 and Salamanca, 2014). Avoidance of overhead irrigation and working in fields when plants are wet (morning dew or after rain) minimizes the bacterial spread from diseased to healthy plants (Goldberg, 2012 and Salamanca, 2014).

Since the pathogen is seed borne, disease management starts with the use of pathogen-free seed (Vincent—Sealy and Brathwaite, 1982; Babadoost, 2012; Goldberg, 2012; Salamanca, 2014). Jarial *et al* (2011) tested various chemicals to be used as seed treatments or foliar sprays against the pathogen and found streptocycline, mancozeb, copper oxychloride, zineb and Bordeaux mixture to be effective against the bacterium under *in vitro* experiments. Seed treatments (dry heat, hot water, sodium hypochlorite, etc.) can reduce the bacterial numbers in the seed, but will not eliminate it completely (Salamanca, 2014). According to Moffett and Wood (1979), seed transmission of the bacterium was eliminated by soaking the infested seed in a 1:20 dilution of commercial hydrochloric acid containing 1 % spreader – sticker for 60 minute while, hot water treatment at 54 to 56°C for 30 minutes and a 1% sodium hypochlorite + 1% spreader-sticker treatment for 40 min greatly reduced the level of seed transmission but did not eliminate it. Jarial *et al* (2015) have reported that a seed dip treatment in a combination of streptocycline (100 ppm) plus copper oxychloride (3000 ppm) for 3.0 h was quite effective in eliminating the bacterium from naturally infected seed.

Frequent foliar application of preventative sprays can help decrease the bacterial population in the field to some extent (Salamanca, 2014). Sinha (1989) reported that 8 foliar sprays of chemicals like plantomycin, paushamycin, streptocycline, Ceresanwet (phenyl mercury acetate), Blitox – 50 (copper oxychloride) and captan were quite effective in manging the disease. Jarial et al (2015) have also suggested a management strategy comprising of a seed treatment with streptocycline (0.01%) plus copper oxychloride (0.3%) and four foliar sprays of same combination at ten days interval along with the removal of diseased plant parts regularly during the cropping season as a useful strategy against bacterial spot of bottle gourd. Although, preventive application of copper can reduce the number or plants infected and the severity of disease development in the field, but has limited efficacy on years with high rainfall, but preventative application of copper formulations is considered more efficacious compared with sprays after the symptoms have developed (Salamanca, 2014). Application of copper compounds during early formation and expansion of fruit may result in substantial fewer symptomatic pumpkins. Copper spray, however, is ineffective once an epidemic is underway (Anonymous, 2012). The development of copper resistance is a growing concern; alternating different modes of action is a tool to prevent and manage copper resistance. Keep in mind that copper formulations should not be applied in solutions having a pH below 6.5. As pH decreases, more copper ions become available and can cause damage in leaves or fruits. However, the efficacy of copper formulations can be impacted at basic pH. A pH range of 6.5 to 8 allows for available copper ions while decreasing the risk of phytotoxicity (Salamanca, 2014).

Conclusion

Bacterial spot caused by *X cucurbitae* is an important disease of cucurbits and has been reported from different parts of the world. The

disease is getting serious day by day in different members of the family cucurbitaceae throughout the world. Till date, only a few aspects of the disease have been studied and further studies are under way. Detailed research is still required for understanding the proper etiology and epidemiology of the disease so as to develop a suitable management strategy for its management world over. In India, only preliminary studies have been conducted on few aspects of disease in cucumber and bottle gourd, though the pathogen attacks other cucurbits also. So, a detailed research is required to be initiated to understand the disease completely according to the prevailing conditions of the country.

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In vitro antibacterial activity as related to antioxidant property of some ethnomedicinal plants

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Abstract

The present study attempted to evaluate *in vitro* antibacterial and antioxidant activities of extracts from some ethnomedicinal herbs and to correlate among the parameters. The antibacterial activity was assayed using agar-disc diffusion method against seven bacterial species. Their total flavonoids content (TFC) and ferric reducing power (RP) were also evaluated. Herb extracts with high TFC exhibited a good antibacterial activity against the bacteria at low concentrations. The Gram-positive bacteria were more sensitive to the tested extracts than the Gram-negative ones. While *Staphylococcus aureus* was maximally inhibited, *Escherichia coli* was most resistant. Against each bacterium, antibacterial activity was positively correlated (r = 0.60–0.87) with TFC of the tested extracts. Positive correlations were also obtained between antibacterial and antioxidant activities (r = 0.60–0.96) as well as between TFC and antioxidant activity (r = 0.91) of the extracts. Thus, antibacterial and antioxidant activities of the tested extracts were closely associated with their flavonoid constituents.

Keywords: Medicinal herb; Flavonoid; Antibacterial; Antioxidant

Introduction

Microbial contaminations from various sources are a major concern throughout the world. With the emergence of new antibiotic-resistant of bacteria, the scenario is becoming quite alarming. A growing demand for natural antimicrobial agents and elimination of synthetic preservatives in food has provided an increased impetus to explore different natural sources. In this context a large number of plant sources, including many medicinal herbs, spices, vegetables and foods, have been explored for their antimicrobial and antioxidant activities (Shan *et al.,* 2007). More recently, medicinal plant extracts were developed and proposed for use in food as natural antimicrobials (Hsieh *et al.,* 2001). Phenolics are a catagory of phytonutrients that exert antimicrobial and antioxidant properties (Shan *et al.,* 2007). They can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Flavonoids, ubiquitous to plants, are active constituents of preparations used from ancient times in curing several diseases (Havsteen, 1983). A strong structure-function relationship exists among these groups of compounds, *viz.* the position and number of hydroxyl groups in the phenolic compounds are possibly related to their relative toxicity to microorganisms, since increased hydroxylation results in increased toxicity (Cowan, 1999). The systemic screening of antimicrobial and antioxidant plant extracts represents a continuous effort to find new compounds with the potential to act against multidrug-resistant spoilage and pathogenic microorganisms.

The Eastern Himalayan region is one of the hotspots in plant biodiversity with a large number of medicinal herbs (Ahmedulla and Nayar, 1999) and in diverse human race too, with a large number of tribes sharing a common habitat. Eventually several plant species of this region have been tagged as medicinal plants and a variety of curative properties have been attributed to the plants used as folk medicines.

Our work aims in resolving (1) *in vitro* antimicrobial profile of the methanolic extracts of five medicinal plants of Darjeeling Himalaya against a panel of bacteria, along with their flavonoid content, and antioxidant activity (reducing power, RP); (2) relationship between antibacterial activity and TFC to understand whether the flavonoids are responsible for the activity; and (3) correlation between antibacterial activity and antioxidant capacity of the extracts, if any.

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Materials and methods

Microorganisms

The bacterial strains used in this study were *Bacillus subtilis* DK-W1, *Escherichia coli* MTCC 118, *Staphylococcus aureus* MTCC 1430, *Listeria monocytogenes* MTCC 839, *Bacillus pumilus* HWC 86, *Bacillus licheniformis* HWC 84 and *Bacillus cereus* HWC 88. All these strains were obtained from the Microbial Culture Collection of the University of North Bengal.

Sampling

Fresh plant specimens (Table 1) were collected in air-tight polyethylene sampling bags and brought to the laboratory as soon as possible. They were cleaned, treated with 8 g $HgCl_2$ l^{-1} ethanol, pressed and dried using blotting paper, and finally mounted onto herbarium sheets. The samples were identified, taking the help from Plant Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal.

Preparation of extracts

Samples of collected plant parts were dried in a hot air oven for 24-48 h at 60°C and pulverized using a waring blender (Bajaj, India). Each of the powders (10 g) was soaked in 10 vol. of methanol (SRL 132977) for 24 h at room temperature with intermittent shaking, and the supernatant decanted. The extraction process was repeated three times, using fresh solvent. Individual extracts were pooled and filtered through a Whatman No. 1 paper, evaporated *in vacuo* at 40°C and lyophilized (Eyela FDU-506 freeze dryer). The lyophilized extracts were stored in a vacuum desiccator at 4°C. Prior to use, the lyophilized extracts were dissolved in methanol.

Disc diffusion assay

Sterilized Whatman No. 1 filter paper discs (5.5 mm) were soaked with different concentrations of herbal extracts (final concentration, 0.1-8.0 mg lyophilized extract $disc^{-1}$) and air dried in a laminar air flow cabinet. Disc diffusion assay was carried out using seven bacterial strains. A loopful of a 24 hold culture was inoculated into tryptone soya broth (HiMedia M011). After 6-8 h. growth on a rotary shaker (200 rpm), the cell concentration was adjusted to 10^8 ml^{-1}, and used for surface seeding using a sterile swab on Mueller-Hinton agar (HiMedia M173) plates. The seeded plates were left to stand for 15 min, impregnated with filter paper discs containing desired concentrations of the herbal extracts and incubated for 18-24 h at 37°C. The assay was carried out in triplicate. A clear zone of inhibition surrounding the discs with a diameter greater than 5.5 mm was considered to be positive.

Assay of flavonoids

The TFC was estimated following the method of Zhinshen et al. (1999). A 400 μl-aliquot of the extract (1 mg ml^{-1}) was added with 30 μl aqueous solution of 50 mg $NaNO_2$ (Merck 61754305001046) ml^{-1}. After incubation for 5 min at 25°C, 30 μl aqueous solution of 100 mg $AlCl_3.6H_2O$ (Merck 80108202501730) ml^{-1} was added, followed after 6 min by addition of 200 μl

Table 1. Plants investigated for potential antibacterial activity

Scientific name	Family	Local name	Part used	Ethnomedicinal use
Cynodon dactylon (L.) Pers.	Poaceae	Dubo	Aerial part	Indigestion, cut and wounds
Enydra fluctuans DC.	Asteraceae	Hincha	Aerial part	Skin disease, liver problem, diabetes and bronchitis
Gloriosa superba L.	Colchicaceae	Bikh-phool	Leaf	Rheumatism, skin disease and leprosy
Leucas indica (L.) W.T.Aiton	Lamiaceae	Dulphe jhar	Leaf	Sinusitis and nasal infections
Ocimum tenuiflorum L.	Lamiaceae	Babari-phool	Inflorescence	Cough and throat infections

aqueous solution of 1 mol l^{-1} NaOH (Merck 61757305001046). The mixture was diluted to 1 ml with water, and the absorbance at 510 nm was read. The TFC value was expressed in terms of mg epicatechin equivalents g^{-1} lyophilized extract, using a standard curve of epicatechin (Sigma E1753).

Assay of reducing power

The ability of the extracts to reduce Fe(III) was assessed according to the method of Oyaizu (1986). A 1.0-ml aliquot of lyophilized extract solution was mixed with 2.5 ml of 0.2 mol l^{-1} phosphate buffer (pH 6.6) and 2.5 ml aqueous solution of 10 g potassium ferricyanide (HiMedia RM1034) l^{-1}. The mixture was incubated for 20 min at 50°C, added with 2.5 ml aqueous solution of 100 g trichloroacetic acid (SRL 204842) l^{-1} and centrifuged at 1200g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 1.0 g $FeCl_3$ (SRL 64765) l^{-1}, and the absorbance was measured at 700 nm. The RP was expressed as mg ascorbic acid equivalents g^{-1} lyophilized extract, using the standard curve of ascorbic acid (SRL 149100).

Statistical analysis

The data were analysed using Minitab version 15.0 (Minitab Inc. State College, PA, USA). The results were expressed as mean ± standard error. Values were considered significant at $P < 0.05$. To assess the relationship between the activities and the flavonoids content, Pearson's correlation coefficients were calculated with 95% confidence level.

Results

Flavonoids content

While the highest TFC was found in *Leucas indica* closely followed by *Ocimum tenuiflorum*, *Cynodon dactylon* had the lowest TFC among the herbs tested (Table 2).

Antibacterial activity

In general, Gram positive bacteria were most susceptible to the majority of the extracts tested (Table 2). On the other hand, the Gram negative bacterium (*E. coli)* was quite resistant as it was inhibited by only two of the five extracts, that too at higher concentrations. The antibacterial inhibitory power of *Enydra fluctuans* was also good as it could control about 71% of the bacteria at a concentration of ≤ 1.0 mg $disc^{-1}$.

Relationship between flavonoids and antibacterial and antioxidant activities

Extracts of *L. indica* and *O. tenuiflorum* with high TFC showed excellent antibacterial activity, while the extract of *C. dactylon* with poor TFC showed no effect (Table 2). The antimicrobial patterns of all the bacteria used were not significantly correlated with TFC of the herbs. Figure 1 shows regression equations and correlation coefficients (*r*) between antibacterial activity and TFC (*P* <

Table 2. Total flavonoids content (TFC), reducing power (RP) and minimum inhibitory concentration (MIC) of the extracts from medicinal herbs*

Scientific name	TFC†	RP‡	MIC (mg lyophilized extract $disc^{-1}$)§						
			Ec	*Lm*	*Bc*	*Bs*	*Bl*	*Bp*	*Sa*
Cynodon dactylon	4.2 ±0.3	22.7±1.88	>8.0	>8.0	7.0	>8.0	>8.0	>8.0	>8.0
Enydra fluctuans	14.1±0.4	822.5±5.27	>8.0	1.0	>8.0	1.0	0.2	0.5	0.2
Gloriosa superba	10.2±0.7	388.1±3.08	3.0	>8.0	7.0	6.0	2.0	7.0	1.0
Leucas indica	25.4±1.5	927.2±2.28	>8.0	7.0	0.5	0.1	0.2	0.2	0.2
Ocimum tenuiflorum	25.0±1.8	868.1±7.40	7.0	5.0	2.0	6.0	1.0	1.0	0.5

* Values indicate mean ± SEM of triplicate sets; † mg epicatechin equivalent g^{-1} lyophilized extract; ‡ mg ascorbic acid equivalent g^{-1} lyophilized extract; § *Ec, Escherichia coli*; *Lm, Listeria monocytogenes*; *Bc, Bacillus cereus*; *Bs, Bacillus subtilis*; *Bl, Bacillus licheniformis*; *Bp, Bacillus pumilus*; *Sa, Staphylococcus aureus*

0.05). The *r* values were between 0.60 and 0.87. *B. cereus* showed maximum correlation closely followed by *B. pumilus*. *S. aureus* and *B. licheniformis* showed moderate correlation, while *B. subtilis* was least correlated. Figure 2 shows the correlation between antibacterial activity and RP (antioxidant activity) of the extracts ($P < 0.05$). The *r* values were between 0.60 and 0.96. *B. pumilus, B. licheniformis* and *S. aureus* had high positive correlations, while *B. subtilis* was moderate and *B. cereus* was the lowest. Figure 3 shows high positive correlation ($P < 0.05$) between flavonoid content and RP.

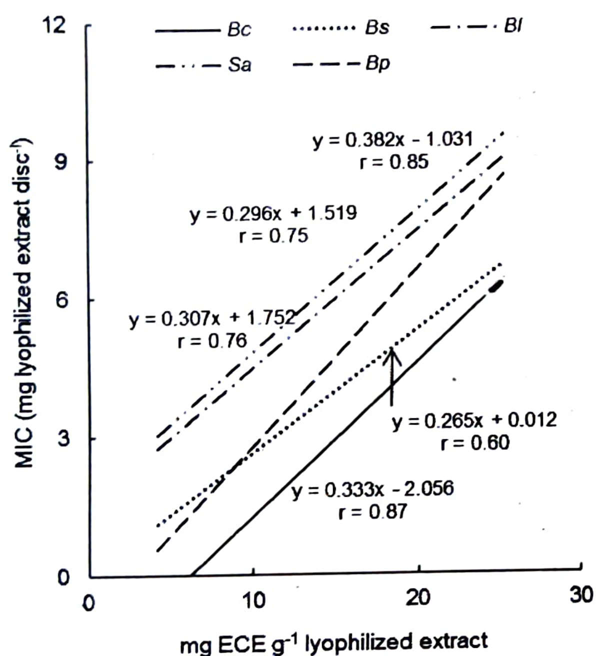

Fig. 1. Regression lines showing relation between total flavonoids content (epicatechin equivalent, ECE) and minimum inhibitory concentration (MIC)

Discussion

Agar disc diffusion method is extensively used to investigate the antibacterial activity of natural substances and plant extracts. The assays are based on the use of discs as reservoirs containing solutions of substances to be examined (Gülçin *et al.*, 2003). Therefore, this method was selected for antibacterial assay. The results show that the Gram positive bacteria are more susceptible than the Gram negative ones. This finding was in consistence with that in literature (López *et al.*, 2005; Shan *et al.*, 2007). This can be explained as there lies a significant difference in the outer layers of the groups of

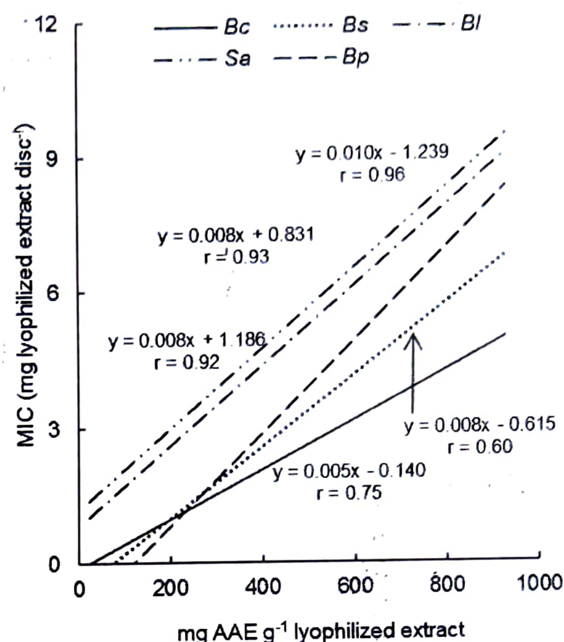

Fig. 2. Regression lines showing relation between antioxidant activity (ascorbic acid equivalent, AAE) and minimum inhibitory concentration (MIC)

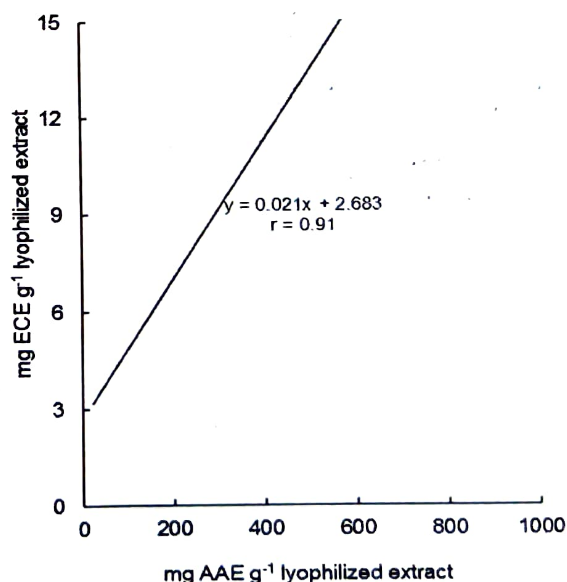

Fig. 3. Regression line showing relation between total flavonoids content (epicatechin equivalent, ECE) and antioxidant activity (ascorbic acid equivalent, AAE) of extracts from five medicinal plants used in the study

bacteria and the Gram negative ones possess an outer membrane and a unique periplasmic space (Duffy and Power, 2001). Antibacterial activity, flavonoids content and antioxidant activities of a number of medicinal herbs have been documented extensively in literature, however, each group had used different assay protocols, different microbial species and varied herb samples. This makes it difficult to establish any relationship between the activities. Shan *et al.* (2007) reported a high positive correlation between the phenolic content and antibacterial and antioxidant activities of medicinal herbs and spices.

The extract of *O. tenuiflorum* inflorescence was the most potent as it inhibited all the bacteria. While both *L. indica* and *G. superba* inhibited 85% bacteria employed in the study, *L. indica* inhibited them at lower concentrations as compared to *G. superba*, which on the other hand controlled *E. coli* at a lower concentration. *Ocimum* spp. are rich in volatile monoterpenes, essential oils, several flavonoid glycosides, flavones, nevadensin, xanthomicrol and gardenin B (Ultee *et al.*, 1999; Grayer *et al.*, 2002). *Leucas* spp. possess several flavonoids such as leucasin, which is reported to have a strong antioxidant potential (Meghashri *et al.*, 2010). Though the flavonoid content of *G. superba* was much lower than that of *L. indica*, its efficient antibacterial activity may be due to the presence of alkaloids, colchicine and colchicoside, which were reported to have antimicrobial properties (Khan *et al.*, 2008).

It is well known that plant polyphenols in general and flavonoids in particular possess antimicrobial and antioxidant activities. Inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolism are the different mechanisms by which flavonoids exert antibacterial actions (Tim Cushnie and Lamb, 2005; Shan *et al.*, 2007). Thus, flavonoids are an important topic of herbal research. Different phytochemical preparations with high TFC have exhibited antimicrobial activity (Rauha *et al.*, 2000). Of course, only a few papers are available that shows correlation between TFC and antimicrobial activities. Flavonoids are also extensively studied for their antioxidant properties. Structure-activity relationship studies of flavonoids have showed importance of the number and location of the phenolic -OH groups for the antioxidant efficacy. A quantitative structure-activity relationship model was developed for correlating the antioxidant capacity of flavonoids with various physicochemical parameters. A positive correlation among the parameters directly indicates that flavonoids are the key factors that control the antibacterial and antioxidant activities of these extracts.

The structure–antioxidant activity relationship of flavonoids is mainly evaluated against different free radicals, but RP assay, which determines directly the reducing capacity of a compound, i.e. 'antioxidant power', has not been given much focus (Firuzi *et al.*, 2005). Studies using RP of flavonoids show that the *o*-dihydroxy structure in the B ring and the 3-hydroxy group and 2,3-double bond in the C ring give the highest contribution to the antioxidant activity. Thus, in the present study, RP was chosen instead of radical-scavenging activity and a good correlation was obtained with total flavonoid content as well as with the antibacterial potential.

From the presented data it is clear that the mere presence of flavonoids in extracts cannot be the sole determining factor for its activity; rather the structures of these compounds are also very important. The orientation of side chains and presence and number of hydroxyl group in the phenolic compounds is the major determining factor for antimicrobial and antioxidant capacity. It is also noteworthy that the action of each phenolic compound against different microbes is also very complicated (Kalemba and Kunicka, 2003). Thus, it is well understood that for better correlation, isolation of the compounds and identification of its structure would only establish its mechanism of action against microorganisms, since in crude extracts a large quantity of diverse polyphenol compounds, with varied potency are present.

The present study demonstrated that several of the extracts contained high levels of phenolics and possessed strong antibacterial and antioxidant activities. They could be a potential source for inhibitory substances against some foodborne bacterial pathogens as well as food spoilage bacteria.

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Management of grey blight disease of Som plants using value added vermicompost with *Glomus constrictum* and *Bacillus altitudinis*

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Abstract
Grey blight disease caused by *Pestalotiopsis disseminata*, is one of the major foliar fungal diseases that constantly affects *Persea bombycina* Kost, a primary host plant of muga silkworm. Under nursery condition, grey blight disease was recorded mostly in S5 and S6 morphotypes of som plants. Vermicompost, PGPR and AMF, alone and in combination were applied for the improvement of the growth of eight morphotypes of som plant as well as to reduce the disease incidence. Growth in terms of height (cm), no. of leaves and no. of branches were studied. Analysis of some major defense related enzymes such as POX, PAL, CHT and GLU was also carried out to check induction of resistance after treatment. Artificial inoculation of som plants under nursery condition with spore suspension of *P. disseminata* was performed and disease progression noted for 7, 14, 21 and 28 days. It was clearly seen that disease progression was slow and less in treated inoculated plants. The results emphasize the fact that application of bioinoculants can be studied in larger scale for the upliftment of the health status of muga host plants.

Keywords: *Persea bombycina*, Vermicompost, PGPR, AMF, foliar fungal diseases.

Introduction

Persea bombycina, commonly called as 'Som' plant is an evergreen tree that belongs to the family Lauraceae. Som is the primary host plant of the silkworm *Antharea assamensis* that produces the golden yellow silk famously called as Muga silk. Cultivation of muga silk is an all year round practice in North Eastern India, mainly Assam. Som plants are mostly grown in the wilds. Hence these plants received very little attention of the scientific community and very less is known about their biochemical and genetic composition. High demand of muga silk has led to the domestication of som plants and rearing of silkworms in closed area. These plants are now-a-days grown significantly in West Bengal, mainly in Coochbehar disrict. Since leaf quality has significant impact on quantity and quality of the silk fiber, for sustaining muga culture it is important to ensure availability of adequate quantity of qualitatively superior leaves.

A major problem in cultivation of healthy som plants that reduces the quantity and quality of the leaves are the various foliar fungal diseases of this plant. One of the major foliar fungal diseases of som plant is grey blight caused by *Pestalotiopsis disseminata* (Das *et al.,* 2010). These diseases are usually controlled through application of various fungicides. But application of fungicides and pesticides cause decline in the quality of the leaves. Use of bioinoculants along with vermicompost have caused decline in the disease incidence as well as improvement in the growth of plants in several cases that has been reported earlier by many researchers in the field (Sahni *et al.,* 2008, Theunissen *et al.,* 2010., Asciutto *et al.,* 2006). Vermicomposting is the simple biotechnological process by which organic material is consumed by earth worms and in the process of their digestion it enhances the process of degradation of the material and converts it into a nutrient-rich end product, called vermicompost. Vermicompost contains most nutrients in plant available forms such as nitrates, phosphates and exchangeable calcium and soluble potassium (Bhattacharjee *et al,* 2015). These help the plant to easily assimilate the required nutrition for its growth and development. Plant growth promoting bacteria (PGPR) can act as an added value. It is considered that as PGPR are soil borne bacteria they can easily mix well with the vermicompost and enhance the effect of the compost. Arbuscular mycorhizal fungi (AMF) are soil microbes forming symbiotic association with plant root system of all most all plant species. Chakraborty *et al* (2013) reported the presence of various AMF spores associated with the different morphotypes of som plants and effect of these

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spores of improvement of plant health. Keeping these findings in mind the following study was undertaken to understand the effect of vermicompost, PGPR as well as AMF, singly as well as jointly on the growth as well as disease establishment of the muga host plant.

Materials and methods

Plant sample

Eight different morphotypes of som plants (S1–S8) were collected from Boko, Assam and maintained under net house condition in Immuno-Phytopathology laboratory, Dept. of Botany, University of North Bengal.

Assessment of disease severity

The disease severity on the leaves was recorded on the basis of a 0-5 rating scale and calculated following the method of Chakraborty *et al* (2014).

Isolation of pathogen and morphological identification

The causative agent of grey blight disease was isolated from infected leaves on PDA slant after surface sterilization with 0.01% $HgCl_2$. The slants were incubated at 28°C for 5-7days till development of black acervulus. The spores were then observed under light microscope for identification of the pathogen. The fungal culture was further maintained in PDA slants.

Preparation and application of bioinoculants

Vermicompost was prepared in plastic beds using organic waste materials collected from the local area. 15-20 cm layer of this waste was covered with another 2-3 cm of dried aquatic plants. *Eisenia foetida*, the earthworm used for vermicomposting was added on the top. The final top layer was made of dried cow dung and the vermin bed was sealed with plastic cover. This set was kept undisturbed for 15-20 days, after which the bed was stirred and shaken to release the organic gas produced during vermicomposting process and for proper mixing of the materials. The compost was ready after 40-45 days when it turned into black light weight powder with no odour. After its completion the earthworms are separated from the final product and the manure was dried and sieved for further use.

The selected PGPR strain (BRHS/ P 73) of *B. altitudinus* was grown in nutrient broth for 48h and then centrifuged at 15000 rpm for 15min. The pellet obtained was re-suspended in sterile distilled water. The optical density of this suspension was measured using a UV-VIS Spectrophotometer at 600nm, to obtain a final density of 1×10^6 cfu/ml. For the preparation of bio-formulation, 10gm of Carboxy methyl cellulose (CMC) was added to 1 kg of talc and the pH was adjusted to 7 by adding calcium carbonate. They were sterilized by autoclaving at 15lbs.p.s.i for 30 mins at 120°C twice. 100ml of bacterial suspension was added to the talc formulation in a mass mixture and mixed for 5 mins. The resultant mixture is packed in polythene bags, labelled and kept at room temperature for further use.

The arbuscular mycorrhizal fungi (AMF) spores obtained from the rhizosphere of Som plants were mass multiplied in maize plant. The spores were washed several times with distilled water. They were then inoculated in roots of 7-10 d-old maize seedlings which were grown in Petri plates. After inoculation they were transferred to black plastic pots (30-cm) having autoclaved soil to eliminate other fungal propagules. The presence of AM spores was confirmed 45 d after inoculation. The maize roots were cut into small pieces and these shredded roots along with soil was added to the rhizosphere of the som plants for treatment.

The selected som plants for study were first treated with 200 g of vermicompost @per pot followed by a mixture of shredded roots and soil containing AMF. 200 g of talc based formulation of PGPR was then added to the treated pots. Aqueous suspension of selected PGPR was also sprayed on the leaves of som plant for three times with an interval of three days after each spray.

Inoculum preparation and application of pathogen

The grey blight pathogen *P. disseminata* was grown in 100ml PDA media in 250 ml flask for 10 days till black acervulus is formed. The spores are then scrapped off the surface of the medium with the help of inoculating media and collected in sterile distilled water. This suspension was filtered through muslin cloth and the filtrate containing $2X10^6$ spores/ml was used further as inoculum source.

This suspension was mixed with few drops of Tween 20 and sprayed onto the leaves of bioinoculant treated as well as untreated potted plants. These plants were kept covered with moist polythene bags for 48hrs to provide adequate temperature for the spores to germinate and establish disease.

Evaluation of Growth

Growth promotion in terms of height, no. of leaves and no. of lateral branches was recorded after every 15 days in both treated and control plants.

Biochemical analyses of leaves

Determination of total soluble protein

Total soluble protein was extracted from the leaves using phosphate buffer (pH 7.2) and was estimated following the method of Lowry *et al.,* 1951.

Estimation of total phenol content of the leaf

Phenol was extracted from the leaves of som plants by boiling in 100% alcohol and crushing in 70% alcohol and filtered. The filtrate is used for estimation of total phenol content following the method of Mahadevan and Sridhar (1978).

Extraction and estimation of defense enzymes

Four major defense enzymes were estimated in the leaves of som plants following the treatments. Phenylalanine ammonia lyase (PAL) enzyme was extracted from the leaves using sodium borate buffer (pH 8.8) and Peroxidase (POX) was extracted from the leaves using phosphate buffer (pH 6.8). Both enzyme extracts were estimated following the method of Chakraborty *et al* (1993). Chitinase and β-1,3-Glucanase both were extracted from leaves using acetate buffer (pH 5). Chitinase was assayed from the enzyme extract following the method of Boller and Mauch (1988) and Glucanase was assayed following the method of Pan *et al* (1991).

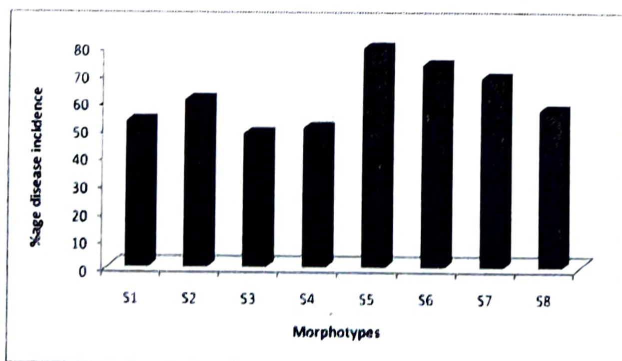

Fig 1: Percent disease incidence in leaves of som plants under nursery condition

Result

Analysis of disease occurrence in nursery condition

Under nursery condition presence of grey blight disease was recorded and percentage disease incidence (PDI) was calculated accordingly. It was recorded that establishment of disease was highest in S5 morphotype and lowest in S3 morphotype (Fig 1). So it can be assumed that morphotype S5 is susceptible to this disease.

Identification of the causal organism

The causal organism of the disease was isolated from the infected leaves in PDA slants. After proper growth, morphological examination showed the growth of white mycelia and presence of black acervulus, typical characters of *Pestalotiopsis* sp. Spores were examined under light microscope and based on the spore characters the organism was identified as *Pestalotiopsis disseminata* (Fig. 2).

Fig 2: (A) Som plant showing symptom of Grey blight; (B-D) *Pestalotiopsis disseminata* - grown in PDA (B), Spores (C) and Germinated spores (D)

Effect of bioinoculants on growth

Application of different bioinoculants was carried out accordingly as outlined in materials and methods. Growth promotion in terms of height, no. of leaves and no. of lateral branches was recorded after 45 days of treatment. In all morphotypes, growth was significantly increased in treated plants in comparison to their respective control plants. Growth was observed to be highest in case of triple treatment of bioinoculants (Table 1, Fig. 3 & 4).

Quantification of different biochemical components of leaf

Total soluble protein was quantified in leaves of control and treated som plants where it was noticed that protein content increased in leaves following the treatments. However the content was more in case of dual treatment of Vermi and PGPR as well as triple treatment when compared to control and single treatment. Total phenol content also increased in leaves following treatment and it was recorded to be highest in S3 and S4 morphotypes. (Fig. 5).

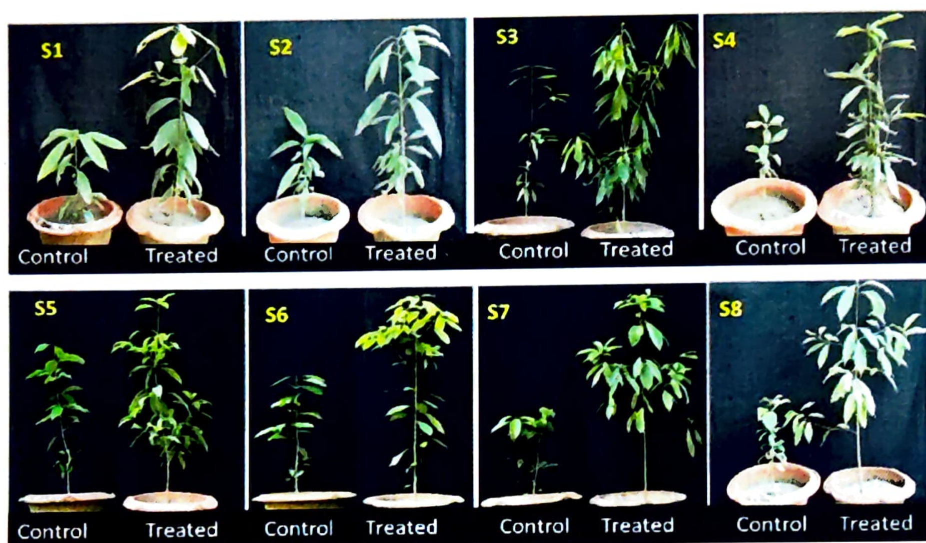

Fig 3: Growth promotion of som plants in glass house conditions after 45days of treatment with *Bacillus altitudinus*

Fig 4: Growth promotion in som plants after 45 days of treatments with bioinoculants (Row 1 Vermi+*B. altitudinus*+ *G. constrictum*, Row 2- Vermi+*B. altitudinus*, Row 3- Vermi, Row 4- *B. altitudinus*, Row 5- *G. constrictum* and Row 6- control)

Table 1: Growth promotion in som plants after 45 days of treatment with bioinoculants

Morphotype	Treatment	Height (cm)	No. of leaves	No. of Branches
S1	Control	29	22	3
	Vermi	54	60	5
	G. constrictum	66	52	6
	B.altitudinus	64	35	5
	B.altitudinus+Vermi	97	35	4
	Vermi+*G.constrictum*+*B.altitudinus*	99	63	8
S2	Control	28	10	1
	Vermi	59	52	4
	G. constrictum	31	42	5
	B.altitudinus	31	43	6
	B.altitudinus+Vermi	69	32	4
	Vermi+*G.constrictum*+*B.altitudinus*	59	33	6
S3	Control	28	25	3
	Vermi	67	77	8
	G. constrictum	69	51	6
	B.altitudinus	67	55	8
	B.altitudinus+Vermi	94	62	6
	Vermi+*G.constrictum*+*B.altitudinus*	67	63	7
S4	Control	26	15	2
	Vermi	50	76	5
	G. constrictum	50	63	5
	B.altitudinus	49	62	4
	B.altitudinus+Vermi	95	65	8
	Vermi+*G.constrictum*+*B.altitudinus*	54	64	9
S5	Control	31	32	3
	Vermi	72	46	5
	G. constrictum	71	57	7
	B.altitudinus	70	55	6
	B.altitudinus+Vermi	93	50	5
	Vermi+*G.constrictum*+*B.altitudinus*	73	53	6
S6	Control	14	16	0
	Vermi	48	55	4
	G. constrictum	55	58	4
	B.altitudinus	56	59	3
	B.altitudinus+Vermi	71	52	4
	Vermi+*G.constrictum*+*B.altitudinus*	60	54	5
S7	Control	30	18	2
	Vermi	48	31	3
	G. constrictum	40	40	4
	B.altitudinus	41	43	5
	B.altitudinus+Vermi	82	55	8
	Vermi+*G.constrictum*+*B.altitudinus*	49	56	9
S8	Control	25	7	0
	Vermi	64	54	4
	G. constrictum	61	60	8
	B.altitudinus	62	63	6
	B.altitudinus+Vermi	68	53	3
	Vermi+*G.constrictum*+*B.altitudinus*	63	55	5
CD (P=0.05)	Treatment	9.308	8.890	1.395
	Morphotype	10.748	10.265	1.611

Assay of different defense enzymes

Four major defense enzymes were studied in leaves of som plants following different treatments. It was observed that PAL, POX, CHT as well as GLU increased in all treatments than in control sets. Highest increased was seen in dual and triple treatment irrespective of morphotypes. (Fig 6)

Artificial inoculation of the pathogen and disease establishment

As disease incidence under nursery condition was highest in S5 followed by S6 morphotype, these two particular morphotypes were taken for further study. After treatment with bioinoculants, treated as well as healthy plants were inoculated with spore suspension of the pathogen and percent disease incidence (PDI) was recorded after 7,14,21 and 28 days of inoculation. It was observed that disease incidence was much less in treated inoculated plants in comparison to untreated inoculated (UI) plants (Table 2). Among the various treatments a consistent decrease in disease incidence was observed in plants treated with PGPR followed by Vermi + PGPR treatment when compared to the untreated inoculated plants. It was seen that disease progression in treated plants were very slow when compared with untreated plants.

Discussion

In the present study growth of all the eight morphotypes was noted following treatment but combined application of vermicompost, PGPR and AMF showed the best result. In an earlier study by Bhattacharjee *et al* (2015) it was found that maximum enhancement of growth and yield was observed in tomato plants treated with vermicompost alone followed by vermicompost along with all microorganisms and then vermicompost with plant growth promoting bacteria (PGPR). An integrated approach by using vermicompost and a strain of *Pseudomonas syringae* (PUR46) containing plant growth promoting traits was adopted under green house condition by Sahni *et al* (2008) where it was recorded that 25% vermicompost and seed bacterization of *Cicer arietinum* resulted in an increased plant growth and also reduced plant mortality against Collar rot disease of chickpea. Pathak *et al* (2003) reported that height of guava plant was stimulated by different bioinoculants (PGPR, VAM, Azotobacter) in combination with farm yard manure as well as vermicompost. VAM inoculation with vermicompost also positively affected number of leaves per plant.

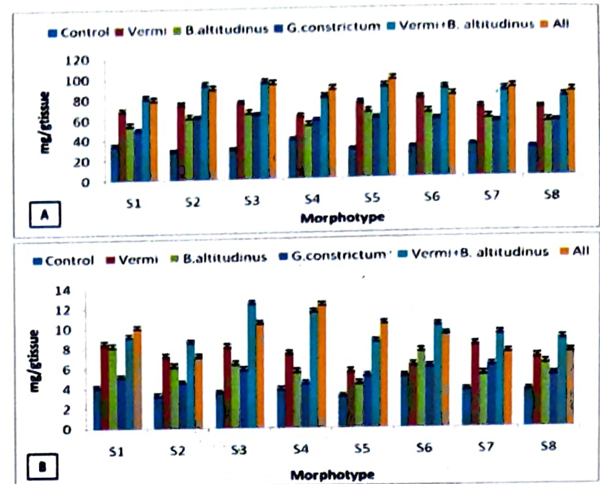

Fig. 5: Total soluble protein (A) and Total phenol (B) contents in som plants following different treatments

Table 2: Percent Disease Incidence (PDI) in S5 and S6 morphotype after artificial inoculation with *P. disseminata*

Treatments	S5 morphotype				S6 morphotype			
	7d	14d	21d	28d	7d	14d	21d	28d
Untreated Inoculated	25.3±0.82	35.6±0.65	68.2±0.42	85.2±0.25	21.2±0.12	29.6±0.22	55.2±0.21	72.5±0.32
Vermicompost	16.2±0.42	25.6±0.62	36.5±0.22	44.2±0.29	14.2±0.11	22.6±0.23	30.5±0.36	39.2±0.35
B. altitudinus	12.5±0.45	20.3±0.35	28.6±0.26	30.5±0.32	17.5±0.24	14.3±0.12	22.6±0.24	28.5±0.36
G. constrictum	18.5±0.84	30.2±0.25	39.6±0.45	48.2±0.25	20.5±0.32	18.2±0.19	29.6±0.24	35.2±0.34
Vermi+*B.altitudinus*	10.3±0.65	15.3±0.28	20.2±0.48	30.5±0.31	12.3±0.15	15.3±0.18	21.2±0.25	25.5±0.24
Vermi+*B.altitudinus*+ *G.constrictum*	15.2±0.52	19.5±0.56	25.5±0.52	29.5±0.36	11.2±0.16	17.5±0.16	22.5±0.32	27.5±0.48

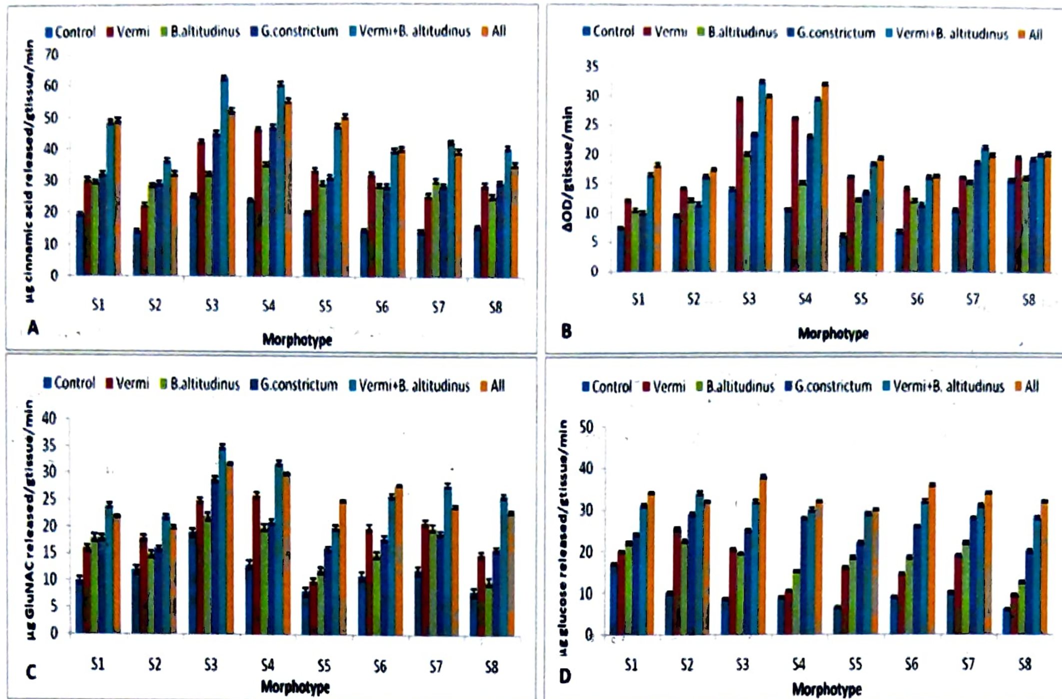

Fig. 6: Activities of defense enzymes (A) PAL, (B) POX, (C) CHT and (D) GLU in leaves of som plants following treatment

Patil (2010) reported that a combined treatment of biofertilizer and chemical fertilizer increased chlorophyll, growth, carbohydrates and proteins content in *Stevia rebaudiana* Var Bertoni compared to control. Similarly, in our study it was observed that treatment with vermicompost and other biofertilizers increased the protein and phenol content in treated plants than in control. However no treatment with chemical fertilizers was carried out.

In an earlier study by Chakraborty *et al*, 2014, it was recorded that dual application of AMF and PGPR increased phenolics as well as other defence enzymes in som plants. Treatment with these bioinoculants also decreased disease incidence in som plants artificially inoculated with *Colletotrichum gleoesporioides*, the causal agent of leaf blight disease of the plant. Similar results were also observed in our study were different defense enzymes were increased following treatment with vermicompost and other bioinoculants. Decrease of disease incidence of grey blight in som plants was also noted in the present study following treatment and artificial inoculation. Therefore it can be concluded that the treatment of som plants with value added vermicompost can lead to sustainable agriculture of such plants related to sericulture.

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Screening of free-living bacteria from the rhizosphere of Jute for their multiple plant growth promoting and antagonistic activity against phytopathogens

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Abstract

Present investigation was undertaken to screen the PGPR isolates from the rhizosphere of Jute for their plant growth promoting and antagonistic activities in the view of an alternative way to chemical fertilizer and hazardous fungicides. A total 76 isolates were isolated from different parts of northern West Bengal and screened for their antagonistic activity against *Macrophomiona phaseolina*. Most promising five isolates were selected for further study and screened for other plant growth promoting and lytic enzyme producing abilities. Out of these, two isolates were Gram positive and rest three Gram negative. All five isolates exhibited several plant growth promoting activities. All five isolates showed IAA and ammonia production whereas four out five showed phosphate solubilization activity. Three PGPR strains exhibited siderophore production and only one isolate showed cyanide production ability. Among the lytic enzymes, chitinase was produced by three isolates. Among them B-3 showed highest degree of chitinase production. Protease was also produced by four strains but amylase and β-1,4-glucanase activity showed by only one isolate, Acti-6. Two isolates B-3 and Acti-6 showed considerable amount of antagonistic activity against three phytopathogens *Macrophomina phaseolina*, *Fusarium oxysporum*, and *F. semitectum* suggesting that Acti-6 and B-3 showed several attributes to be the potent strains of PGPR and can be used as biofertilizer as well as biocontrol agents.

Keywords: PGPR, Jute, Antagonistic activity, Phytopathogen

Introduction

Improper use of chemical fertilizers and pesticides in search of high crop yield and quality is present practice in agriculture after the green revolution. But this approach is costly and imposes threat to the environment and human health (Xu *et al.* 2014). For the last few years scientists are searching an alternative and sustainable way to overcome this problem. Application of plant growth promoting bacteria may be the way. Plant growth promoting rhizobacteria (PGPR) are soil bacteria those lives in vicinity of root system can produce beneficial effect on plant health. The PGPR can promote the plant growth either directly or indirectly but the exact mechanisms by which they can act beneficially on plant growth have not been fully elucidated. The direct mechanisms of plant growth promotion involve the synthesis of substances by the bacterium which facilitates the uptake of nutrients from the environment (Glick *et al.*, 1999). The direct growth promoting activities are as follows i) nitrogen fixation ii) solubilization of phosphorus iii) production of phytohormones such as auxins, cytokinins, gibberellins and iv) lowering of ethylene concentration (Kloepper *et al.*, 1989; Glick, 1995; Glick *et al.*, 1999). The indirect mechanism of plant growth promotion by PGPR include i) antibiotic production ii) depletion of iron from the rhizosphere iii) synthesis of antifungal compound and fungal cell wall lysing enzymes such as cellulase, protease, antibiotics and cyanide iv) competition for sites on roots and induced systemic resistance (Kumar *et al.* 2012, Kavamura *et al.*, 2013, Xu *et al.* 2014).In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Alcaligens, Enterobacter, Arthobacter, Bacillus, Burkholderia,* and *Serratia* have been reported to enhance plant growth (Kloepper *et al.*, 1989; Glick, 1995).

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Jute (*Corchorus olitorius* L. and *C. capsularis* L.) is one of the important commercial fiber crop of India and Bangladesh. In India jute mostly grown in its eastern part with area of 0.91 million hectares and production 11.82 million bales (one bale = 180 kgs.). Recently, jute has attracted the concentration due to its eco-friendliness. Jute crop suffers from several fungal, bacterial and viral diseases but the most devastating one is the stem rot of jute, caused by *Macrophomina phaseolina* (Tassi) Goid, affecting both quality and yield (De, 2014). Hence, the present study was intended to isolate and characterize PGPR strains from the rhizosphere of Jute having PGP and antagonistic traits so that they can be exploited as potential bioinoculants.

Materials and Methods

Collection of soil samples from Jute rhizosphere

The present investigation was under taken with an objective to select promising native PGPR strains which can promote the growth as well as can induce resistance against *Macrophomina phaseolina*, the most devastating pathogen of Jute (*Chorchorus sp.*). For this purpose, soil samples were collected from different agricultural fields of the northern part of the West Bengal, India. The samples were placed in plastic bags and stored at 4°C in the Laboratory, Department of Botany, University of North Bengal for further study.

Isolation and characterization of bacteria

Bacteria were isolated from the rhizosphere of healthy jute plants (*C. olitorius*). Ten grams of soil particles loosely adhering to the roots were collected. The soil suspension was prepared by dissolving the soil sample in 100 ml of sterile distilled water using magnetic stirrer for 1 h. Then the upper light brown colored layer was pippeted out and serial dilutions were made. Appropriate dilutions were spread over the nutrient agar medium and incubated at 37° C for 24-48 hrs. Colonies with different morphological appearances were selected from the countable plates and sub cultured in nutrient agar slant for their further use. For the long term use bacterial strains were maintained in 50% glycerol at -20° C. A total 76 bacterial isolates were isolated and used in present study.

The selected bacterial isolates were examined for their morphological features in terms of colour, shape, size, surface and gram staining etc.

Screening for multiple plant growth promoting activities

All 76 rhizospheric isolates were first screened for their antagonistic activity against *M. phaseolina* and promising isolates were further analysed for their other plant growth promoting activities.

Phosphate solubilizing activity

The phosphate solubilising test was done in the solid medium. Pikovskaya's medium was used for screening of phosphate solubilization (Pikovskaya, 1948). Agar plates were prepared and the bacterial strains were individually spot inoculated at the center of the plates followed by incubation for 5 - 6 days. The plates were observed for clear zone around the colony and the diameter of the clearing zone was recorded.

IAA production

IAA production by bacterial isolates was determined following the methods of Gordon and Weber (1951). Luria Bertani broth medium amended with 0.1 mM tryptophan was inoculated with the isolated bacteria. They were incubated for 24 h at 30°C on rotary shaker and the cultures were centrifuged at10,000 g for 15 min. Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al.* (1999). For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature.

Siderophore Production

The selected isolates were characterized for siderophore production following standard method (Schwyn and Neiland, 1987) using blue

indicator dye, chrome azurol S (CAS). For preparing CAS agar, 1 L, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). With constant stirring this solution was added to 72.9 mg hexa-decytrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petri plates. The plates were inoculated with the bacteria and incubated at 30°C for 10-15 days till any change in the color of the medium was observed.

HCN production

Hydrogen cyanide (HCN) production was evaluated by streaking the bacterial isolates on Luria Bertani agar medium amended with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plate. The plates were then sealed air-tight with parafilm and incubated at 30°C for 48 h. A colour change of the filter paper from deep yellow to reddish-brown colour was considered as an indication of HCN production (Bakker and Schippers, 1987).

Production of Ammonia

The ability of bacterial isolates for the production of ammonia were tested according to Cappuccino *et al.* (1992). For the production of ammonia bacterial isolates were grown in 10 ml peptone water in each tube separately and incubated for 48-72 h at 30 ± 2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production.

Extra cellular enzyme activities

Catalase activity

Catalase activity of selected isolates were performed by adding 3-4 drops of hydrogen peroxide (H_2O_2) to 48 h old bacterial colonies. The appearance of effervescence indicated catalase activity (Schaad NW, 1992).

Chitinase production

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 10 g colloidal chitin with 20 g of agar in M9 medium (Na_2HPO_4 0.65 g, KH_2PO_4 1.50 g, NaCl 0.25 g, NH_4Cl 0.50 g, $MgSO_4$ 0.12 g, $CaCl_2$ 0.005 g and distilled water 1 L; pH 6.5).

The CDA plate was spot inoculated with organism followed by incubation at 28°C for 7-10 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production (Robert and Selitrennikoff; 1988).

Protease activity

The qualitative assay for protease production was performed on sterile skim milk agar plates (Panc. digest of casein 5.0, Yeast extract 2.5, Glucose 1.0, Agar 15.0, Distilled water 1000 ml, Skim milk 7% was added as inducer). Isolates were spot inoculated and followed by incubation at 30°C and plates were examined for development of clear zones around colonies (Walsh *et al.* 1995).

Amylase activity

The bacterial isolates were spot inoculated on starch agar (Beef extract 3.0, Peptone5.0, soluble starch 2.0, Agar 15.0, Distilled water 1 lit.) medium plates and incubated at 30°C for 48 h. At the end of incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Production of colourless zone surrounding colonies was considered positive for the production of amylase (Shaw *et al.*1995).

β-1,4-glucanase activity

Isolates were inoculated by spotting on the plates having cellulose powder as a sole source of carbon and incubated at 30 ± 2° C for 3-5 days. These plates were examined

for development of clear zones around colonies (Rangel-Castro *et al.*2002).

Antagonistic activities against phyto-pathogens

All five isolates were tested for their antifungal activities against *Macrophomina phaseolina*, *Fusarium oxysporum*, and *F. semitectum* on PDA plates. Isolates were inoculated on the surface of agar plate 2 cm away from fungal disc. Antagonist activity was observed after incubation at 28 ± 1^0 C up to 7 days.

Results

Isolation and characterization of bacteria

A total of 76 bacteria were isolated from various jute growing fields of the northern part of West Bengal. These isolates were evaluated for their antagonistic and plant growth-promoting traits. Out of 76 the best five potential bacterial strains showing antagonistic and PGP activities were selected for characterization.

The morphological characteristics of Acti-2, Acti-3, Acti-6, B-3 and PKV+ varied widely. Acti-2, Acti-3 and PKV +produced smooth, shiny, convex colony with entire margin whereas Acti-6 and B-3 produced waxy, non-elevated colony with undulated margin (Table 1).

Screening for multiple plant growth promoting activities

Phosphate solubilizing activity

All the isolates except B-3 showed ability for phosphate solubilization on Pikovskaya medium with different efficacy. Out 4 strains Acti-3 showed maximum degree of phosphate solubilization. The phosphate solubilizing activity of the isolates indicates that they are able to secrete the organic acids that chelate the cations, converting insoluble phosphate into soluble form and thus available for plants.

IAA production

All the five strains showed development of brown to pink colour indicating their capability of IAA production. IAA is the most physiologically active form of auxin. Recent investigations on auxin synthesizing rhizobacteria demonstrated that the rhizobacteria can synthesize IAA from tryptophan by different pathways, although the general mechanism of auxin synthesis was basically concentrated on the tryptophan-independent pathways (Spaepen *et al.* 2007). Among the jute rhizospheric isolates PKV+ showed highest IAA production followed by Acti-3, Acti-2 and Acti-6.

Siderophore Production

Siderophore production was determined blue CAS agar medium. Formation of yellow to orange zone around the colonies of isolates Acti-6, Acti-2 and B-3 was observed, indicating their capacity to chelate iron from the surrounding

Table.1 Morphological and Microscopic characters of PGPR isolates

Isolate Code	Shape	Gram stain	Colour	Surface	Margin	Pigmentation
Acti-2	Bacilli	Negative	White	Smooth, Shiney	Entire	Non-pigmented
Acti-3	Bacilli	Negative	White	Smooth, Shiney	Entire	Non-pigmented
Acti-6	Bacilli	Positive	Off white	Rough, waxy appearance	Irregular	Non-pigmented
PKV+	Bacilli	Negative	White	Smooth, Shiney	Entire	Non-pigmented
B-3	Bacilli	Positive	White	Rough, waxy appearance	Irregular	Non-pigmented

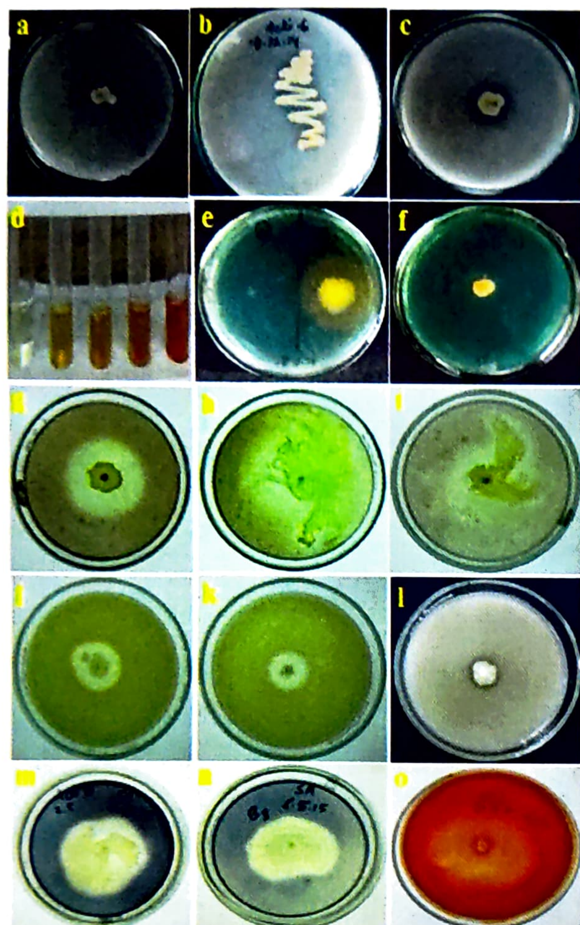

Fig. 1 Phosphate solubilization-Acti-3 (a), Acti-6 (b), PKV+ (c); IAA production-control, B-3, Acti-6, Acti-3, Acti-2 & PKV+ (d); siderophore production-Acti-2 (e) & Acti-6 (f); Chitinase production-B-3(g), Acti-6 (h) & Acti-2 (i); Protease production-Acti-6 (j), B-3 (K) & Acti-3 (l); Amylase production-Acti-6 (m) & B-(n); β- 1, 4-glucanase-Acti-6.

medium and thereby depriving the pathogens.

HCN production

Ability for hydrogen cyanide synthesis was observed for selected five isolates. Among the isolates, only Acti-6 showed the HCN production ability.

Production of Ammonia

The production of ammonia observed in all the five isolates. Ammonia production by the plant growth promoting bacteria helps to influence plant growth indirectly.

Extra cellular enzyme activities

Chitinase production

The spot inoculated CDA plates were incubated at 28°C for 7-10 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production. The plates of Acti-6, Acti-2, B-3 showed halo zone around the colonies indicating their capability of secretion extracellular chitinase. It was observed that no extracellular chitinase was secreted by Acti-3 and PKV+ even when grown on chitin amended media.

Protease activity

Proteolytic enzyme production was detected as formation of a clear zone around the colony on skim milk agar. All the isolates showed variable degree of protease activity except PKV+. Among the isolates Acti-6 showed high production of extracellular protease.

Amylase activity

Amylase activity was determined by spot inoculation of isolates on starch agar plates. After 72 to 96 hrs. of incubation the plates were flooded with Iodine solution for1min and the appearance of clear zone surrounding the colony indicates positive for starch hydrolysis test. Among the isolates Acti-6 and B-3 showed amylase activity

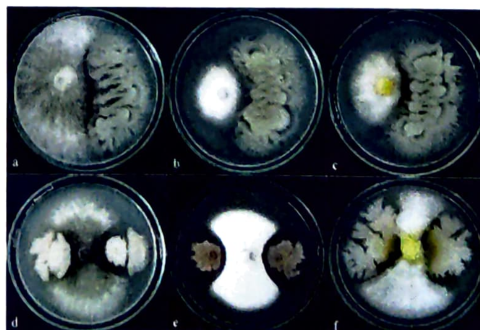

Fig. 2 Antagonistic activity of Acti-6 (a,b,c) and B-3 (d,e,f) against *M. phaseolina* (a), *F. oxysporium* (b) & *F. semitectum.*

Table.2 Plant growth promoting characteristics and lytic enzyme production traits of PGPR strains

Isolate code						Lytic Enzyme Production			
	Phosphate solubilization	IAA Production	Siderophore Production	HCN Production	Ammonia Production	Chitinase	Protease	Amylase	β- 1, 4-glucanase activity
Acti-2	++	++	+	-	+	+++	+	-	-
Acti-3	+++	++	-	-	+	-	+	-	-
Acti-6	+	+	+	+	+	++	+ + +	+	+
PKV+	+ + +	+ + +	-	-	+	-	-	-	-
B-3	-	+	+	-	+	+ + +	+ +	+	-

β- 1, 4-glucanase activity

β- 1, 4-glucanase activity of the isolates were determined in minimal medium plates using cellulose as only carbon source. Only Acti-6 showed a halo zone around the colony after the addition of congo red indicating secretion of extracellular β- 1, 4-glucanase. Plant growth promoting traits and extracellular enzyme secretion by the isolates have been summarized in Table 2 and Figure 1.

Antagonism against pathogens

Antagonistic activity of the bacterial isolates were evaluated in terms of inhibition zone diameter as an indicator of the reduction in growth of 3 fungal pathogens. Among the isolates, Acti-6 and B-3 showed antagonistic activity against *M. phaseolina, Fusarium oxysporium* and *F. semitectum* considerably (Fig. 2).

Discussion

Many types of microorganisms are known to inhabit soil and plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability (Geetha *et al.* 2014). Plant growth promoting rhizobacteria (PGPR) perform important functions in promoting plant growth and sustaining plant health. Direct plant growth promotion by microbes is based on improved nutrient acquisition by solubilizing insoluble phosphate and hormonal stimulation (Walia *et al.* 2014). Varied mechanisms are involved in the suppression of plant pathogens which are often indirectly connected with plant growth. Beneficial plant–microbe interactions have led to development of microbial inoculants for use in agricultural biotechnology (Berg 2009).

In the present study, a total of 76 isolates were isolated from the rhizospheric soil of jute (*C. olitorius*). All the isolates were screened for their antagonistic activity against *M. phaseolina,* most devastating pathogen of jute and other plants. Out of 76 five isolates (Acti-2, Acti-3, Acti-6, B-3 and PKV^{+}) showed varied degree of antagonism against *M. phaseolina* and the isolates were screened for their other *in vitro* plant growth promoting activities. All the isolates except B-3 showed ability for phosphate solubilization on Pikovskaya medium. Among the PGPR strains Acti-3 showed maximum degree of phosphate solubilization, which is due the production of organic acid, thus converting insoluble phosphate into soluble form and available for plants. The mechanism of plant growth promotion by PGPR includes the production of plant hormones. In present study isolates were screened for the production of IAA, which is most active form of auxin. All the five strain showed development of brown to pink colour indicating their capability of IAA production. Isolates Acti-6, Acti-2 and B-3 also showed the siderophore producing capacity. The production of siderophore like iron chelating

compound is an important criterion for plant growth promoting rhizobacteria. They chelate the iron from the surrounding medium and soil, rendering it unavailable to pathogens. Rhizobacteria can inhibit phytopathogens by the production of hydrogen cyanide (Bloemberg and Lugtenberg 2001). HCN is known to inhibit electron transport, and the energy supply to the cell is disrupted leading to the death of the organisms. It also inhibits the proper functioning of different enzymes such as of cytochrome oxidase (Gehring *et al.*1993). Acti-6 showed HCN production ability and thereby it can be considered as a potent antagonist against plant pathogens. Isolates were also screened for protease activity. Out of five isolates, 4 showed protease activity. Very high protease activity was exhibited by Acti-6. Siddiqui *et al.* (2005) reported the biocontrol activity of *Pseudomonas fluorescens* CHA0 by extracellular protease. Three PGPR isolates, Acti-6, Acti-2 and B-3 showed significant amount of chitinase production. Several workers reported biological control of plant pathogen by chitinase producing microorganisms (Raaijmakers *et al.*2006; Kamal *et al.* 2008). Acti-6 and B-3 showed antagonistic activity against *M. phaseolina, Fusarium oxysporium* and *F. semitectum* considerably.

The current study has been undertaken as a search of an alternative to chemical fertilizer and fungicide. A group of promising rhizobacterial isolates was screened through *in vitro* and their plant growth promoting properties. Based on our results we concluded that Acti-6 and B-3 showed several attributes to be the potent strains of PGPR and can be used as biofertilizer as well as biocontrol agents. Future research in this direction is required to harness their potential as bio-inoculants for sustainable agriculture.

Acknowledgement

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Larger Fungi from the Pir Panjal Himalayan range in Poonch district of Jammu and Kashmir state, India

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Abstract
Nine macrofungal species belonging to seven families collected from temperate forests of district Poonch, Jammu and Kashmir have been described. All these species constitute new report of their occurrence from the study area.

Keywords: Poonch, Mycodiversity, Taxonomy, Edible

Introduction

The State of Jammu and Kashmir falls in the great north-western complex of the Himalayan ranges (between 32° 17′ N–36° 58′ N latitude and73° 26′ E–80° 30′ E longitude) and possess great snow-clad mountains, altitudinal variation, diverse geological structures, different climatic zones, antecedent drainage and rich temperate flora and fauna. The presence of interesting mycodiversity in this Himalayan region has been attracting many enthusiastic investigators over the years. From Jammu and Kashmir, different researchers have contributed to the study of mushroom flora and a total of about 340 macrofungal species have been reported so far (Abraham *et al.,* 1981, 1984; Abraham and Kaul, 1985; Watling and Abraham, 1986, 1992; Abraham, 1991; Dar *et al.,* 2009; Wani *et al.,* 2010; Beig *et al.,* 2011; Kumar and Sharma, 2008, 2009, 2011a, b, c; Sharma and Sharma, 2012; Sharma *et al.,* 2012; Dorjey *et al.,* 2013a, b; Kour *et al.,* 2013; Kumar *et al.,* 2014; Kour *et al.,* 2015) and still rich mycoflora is hidden in these regions which need to be explored. Our laboratory is also engaged in survey of macrofugal diversity of the state for the last ten years. In this communication, we are reporting nine mushroom species collected from the temperate forests of district Poonch.

Materials and Methods

Collections were made from different locations of district Poonch of Jammu and Kashmir during the monsoon season. The specimens were photographed in the field and extensive notes were made from the fresh fruit bodies before they were dried. Colour terms and notations are from Ridgway (1912). The microscopic details were studied from thin sections made from dried specimen, revived in 5% KOH and stained in 1% Congo red and Melzer's reagent. The spore Quotient (Q) was obtained by mean spore length divided by spore width. Identification was carried out by making use of taxonomic keys, field manuals and help of mushroom experts. The examined specimens have been deposited in the Herbarium of Botany Department, University of Jammu with accession numbers (HBJU).

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Study area

District Poonch (73° 58′- 74° 35′ E longitude and 33° 25′- 34° 01′ N latitude), Jammu and Kashmir, from where the present collections have been made fall in the temperate climatic zone with an altitudinal range of 600-4,750 m. It is characterised by the forests comprising of *Pinus roxburghii, P. wallichiana, Abies pindrow, Cedrus deodara, Juniperus communis, J. recurva, Picea smithiana, Taxus wallichiana, Juglans regia, Quercus semecarpifolia, Populus ciliata, Platanus orientalis, Pyrus pashia, Prunus padus, P. armeniaca, Eucalyptus globulus, Vitex negundo, Morus alba* and *Dalbergia sisoo.* Apart from this, medicinally important herbs and shrubs including *Adhatoda vasica, Asparagus sp., Atropa belladonna, Berberis sp., Dodonae viscosa, Ocimum sanctum, Podophyllum hexandrum, Woodfordia floribunda and*

Zanthoxylum alatum etc. are also found in the area.

Results and Discussion

1. ***Lepiota sistrata*** (Fr.) Quel., *Mem. Soc. Emul. Montbeliard, Ser.* 2 5: 231 (1872)

Synonymy: *Agaricus sistratas* Fr., *Syst. Mycol.* (Lundae) 1: 24 (1821)
Lepiota sistrata (Fr.) Quel., *Mem. Soc. Emul. Montbeliard*, Ser. 2 5: 231 (1872)

Pileus: 1.8-3.2 cm in diameter, white at margins and yellow in the centre, globose when very young and campanulate at later stages, hemispherical when mature; **Gills:** creamy white, sub-distant, unequal; **Stipe:** 0.3 cm in diameter and 4.5-6.0 cm in length, brittle; **Veil:** present in the form of cortina in young stages; **Basidiospores:** 4.0-7.2 x 4.0-4.8 µm, a_vL= 5.6, a_vW= 4.4, Q= 1.0-1.5, smooth, thick walled, uni- to multiguttulated, apiculate, broadly elliptical; **Apiculus:** 0.8-1.6 µm in length; **Basidia:** 15.2-27.2 x 5.6-8.0 µm, clavate, hyaline; **Sterigmata:** 3.2-4.8 µm long, four in number; **Pileus cuticle hyphae:** hyaline, 8.0-18.0 µm wide, septate, hyaline, branched; **Inflated hyphae:** 28.0-32.0 µm wide; **Pileus context hyphae:** 8.0-18.0 µm wide, hyphae branched, septate, hyaline; **Pileocystidia:** clavate to pyriform, 36.0-88.0 µm long; **Stipe hyphae:** 8.0-26.0 µm wide, hyaline, septate, less branched, clamp connections absent.

Collection examined: Jammu and Kashmir, Poonch, Dara Dullian, fasciculate, humicolous, in mixed forests of *Quercus semecarpifolia* and wild *Punica granatum*, Harpreet Kour and Y. P. Sharma, HBJU 214, October 2012.

Distribution: Reported on ground of botanical Garden, Saharanpur, U.P (Hennings, 1901).

2. ***Panaeolus campanulatus*** (L.) Quel., *Mem. Soc. Emul. Montbeliard*, Ser. 25: 151 (1872)

Synonymy: Agaricus *campanulatus* Fr., Syst. mycol. (Lundae) 1: 295 (1821)

Pileus: 1.0-2.5 cm long and 1.0-2.5 cm wide, grayish-black, conico-convex; **Gills:** black in colour, free; **Stipe:** 6.5-8.0 cm long and 0.3 cm wide, equal, brown; **Odour:** agreeable; **Basidiospores:** 12.0-16.0 x 10.0-12.0 µm, hyaline, broad at centre narrow towards both the ends, double walled, smooth; **Basidia:** 6.4-12.8 x 4.8-7.2 µm, hyaline, clavate, small in size, guttulated; **Sterigmata:** 2 to 4 in number,1.6-3.2 µm long; **Pileus hyphae:** hyaline, septate, branched, clamp connections absent; **Pileocystidia:** 32.0-36.0 µm wide and 40.0-108.0 µm long, hyaline; **Stipe hyphae:** hyaline, septate, unbranched, 10.0-24.0 µm wide.

Collection examined: Jammu and Kashmir, Poonch, Krishna Ghati, single, gregarious, humicolous, Harpreet Kour and Y.P. Sharma, HBJU 225, August 2012.

Distribution: On ground of botanic garden Saharanpur, U.P (Hennings, 1900; Bose, 1920) and in forests of Kashmir (Beig et al., 2011).

3. ***Lycoperdon pedicellatum*** Peck, *Bull. Buffalo Soc. Nat. Hist.* 1(2): 63 (1873) [1873-1874]

Synonymy: *Bovistella pedicellata* Lloyd, *Mycol. Writ.*2 (Letter 23): 284, pl. 88, fig. 5-10 (1906)

Carpophore: 1.5-3.4 cm in diameter, dresden brown (17'.O-Y.k) in colour, soft, with aperture at apex; **Stipe:** 4.5 cm long and 1.6 cm in diameter, fused at base to form groups, creamish; **Exoperidium:** dark brown with an apical pore; **Basidiospores:** globose, 4.0-5.6 µm in diameter, echinulate, double walled, pale yellow in Congo red; **Pedicel:** 2.4-9.6 µm long, **Capillitium threads:** hyaline to light yellow in color, branched; **Exoperidial hyphae:** 4.0-7.2 µm wide, septate, double walled, branched; **Inflated hyphae:** 10.4-28.8 x 11.2- 22.4 µm, thin walled.

Collection examined: Jammu and Kashmir, Poonch, Kanuyian, fasciculate, gregarious, humicolous, Harpreet Kour and Y.P. Sharma, HBJU 217, September 2012.

Figure 1: a= Fruit bodies of *Lepiota sistrata* b= *Lycoperdon pedicellatum* in natural habitat c= Fruit bodies of *Lactarius deliciosus* d= Sporophores of *Panaeolus campanulatus* e= *Lepiota procera* growing in nature f= Undersurface of fruit body of *L. procera* showing lamellae and annulus.

Figure 2: a= Sporocarps of *Suillus granulatus* b= *Amanita vaginata* in natural habitat c= Carpophores of *Astraeus hygrometricus* d= Carphophores of *A. hygrometricus* showing reticulate pattern on exoperidium e= Carpophores of *Scleroderma citrinum* in natural habitat.

Distribution: Earlier reported from conifer dominated forests of Kashmir (Beig *et al.,* 2011).

4. *Scleroderma citrinum* Pers., *Syn. Meth. Fung.* (Gottingen) 1: 153 (1801)

Synonymy: *Scleroderma vulgare* Hornem., *Syst. Mycol.* (Lundae) 3: 46 (1829)

Carpophore: 1.1-1.6 cm wide, light brown in colour with reticulate pattern on it, on maturity cracks into lobes to form an irregular pore, profusely branched rhizomorphs present; **Gleba:** dark black (in preservative); **Stipe:** short; **Basidiospores:** 6.4-10.4 x 6.4-9.6 µm, a_vL= 8.4, a_vW= 8.0, Q= 1.0-1.1, light brown (in Congo red), yellowish green in KOH and iodine, echinulated, double walled; **Pedicel:** absent; **Exoperidial hyphae:** 4.8-8.8 µm in width, septate, branched; **Endoperidial hyphae:** hyaline, thin, 4.0- 5.6 µm wide.

Collection examined: Jammu and Kashmir, Poonch, Islamabad, humicolous, Harpreet Kour and Y.P Sharma, HBJU 233, July 2012.

Distribution: Recorded from the forests of Kashmir (Beig *et al.,* 2011).

5. *Lepiota procera* (Scop.) Gray, Nat. Arr. Brit. Pl. (London) 1: 601 (1821)

Synonymy: *Agaricus colubrinus* Bull., *Herb. Fr.*2: tab. 78 (1782) [1781-82]
Agaricus procerus Scop., *Fl. carniol.*, Edn 2 (Wien) 2: 418 (1772)

Pileus: creamy white, 6.5-10.5 cm wide, soft spongy in texture, convex and sub- umbonate with brown appressed scales on it; **Gills:** creamy white, crowded, sinuate; **Stipe:** creamy white, 15.0-20.0 cm long and 1.5-2.5 cm wide, solid, brittle, equal, centric; **Basidiospores:** hyaline, ovate, thick walled, apiculate, 10.4-15.2 x 8.0-11.2 µm, a_vL= 12.8, a_vW= 9.6, Q= 1.3-1.4; **Basidia:** clavate, hyaline, 29.6-40.0 x 11.2-14.4 µm; **Sterigmata:** 2-3 in number and 4.8-6.4 µm long; **Pileus hyphae:** hyaline, septate, branched occasionally, 6.0-10 µm wide; **Stipe hyphae:** hyaline, septate, branched, clamp connections present, 7.2-28.0 µm in width.

Collection examined: Jammu and Kashmir, Poonch, Krishna Ghati, gregarious, fasciculate, humicolous, mixed forest of *Pinus roxburghii* and *P. wallichiana*, Harpreet Kour and Y.P. Sharma, HBJU 213, July 2012.

Edibility: Edible in the study area.

Distribution: Earlier reported from Calcutta (Banerjee, 1947); Saharanpur (U.P) (Chopra and Chopra, 1955).

6. *Amanita vaginata* Encycl. Meth. Bot. (Paris) 1: 109 (1783)

Synonymy: *Agaricus plumbeus* Schaeff., Fung. Bavar. Palat. 4: 37 (1774)
Amanita vaginata f. battarrae (Boud.) Vesely, Annls. Mycol. 31(4): 279 (1933)

Pileus: 3.5-4.5 cm wide, plane, smooth, grayish white with white patches on pileus; margins plicate-sulcate, thin, crenate, entire; **Gills:** free, close to sub-distant, entire, whitish; **Stipe:** 4.5-6.5 x 0.7-0.9 cm, central, cylindrical, smooth, white, slightly bulbous at the base, volva forming a cup at the base of stipe, soft, brittle, solid, becoming hollow at maturity; **Spore print:** white; **Annulus:** absent; **Basidiospores:** globose to slightly ellipsoidal, 8.0-11.2 x 7.2-12.0 µm, a_vL= 9.6, a_vW= 9.6, Q= 1.1-0.9, smooth, oil drops present, biguttulate, hyaline (3% KOH), amyloid with prominent apiculus; **Basidia:** clavate, 30.4-51.2 x 17.6-19.2 µm, sterigmata 2 to 4 in number, ranges upto 3.2 µm long; **Pileus hyphae:** 2.4-4.8 µm wide, thick-walled; **Stipe hyphae:** 4.8-8.0 µm wide, septate, hyaline, unbranched.

Collection examined: Jammu and Kashmir, Poonch, Kanuyian, forests predominated by *Quercus semecarpifolia*, solitary, humicolous, Harpreet Kour and Y.P. Sharma, HBJU 219, September 2012.

Edibility: Not eaten in the study area. However its edibility has been reported across the world

(Christensen, 1955; Purkayastha and Chandra, 1985).

Distribution: On soil from Chattisgarh, Himachal Pradesh, Assam, Nagpur, Uttar Pradesh, Tamil Nadu and from Kashmir region (Trivedi, 1972; Ghosh *et al.,* 1974; Sathe and Sasangan, 1977; Watling and Gregory, 1980; Shukla *et al.,* 2009).

7. *Astraeus hygrometricus* (Pers.) Morgan, J. Cincinnati Soc. Nat. Hist.12: 20 (1889)

Synonymy: *Astraeus hygrometricus* f. decaryi (Pat.) Pat., *Mem. Acad. Malgache* 6: 35 (1928) (1927)
Lycoperdon stellatus Scop., *Fl. carniol.*, Edn 2 (Wien) 2: 489 (1772)

Exoperidium: 1.0-2.5 cm long and 0.8-2.0 cm wide at the base, splitted into 8-12 rays, hygroscopic, pale yellow, with reticulate pattern; **Endoperidium:** forming spore sac, open by irregularly torn aperture, sessile, depressed; **Gleba:** dark brown; **Basidiospores:** yellow greenish (in Congo red), 8.0-15.2 x 6.4-12.8 µm, a_vL= 11.6, a_vW= 9.6, Q= 1.2, broadly ellipsoidal, monoguttulated, thick walled, ornamented, faint concentric rings seen in the centre; **Capillitium threads:** hyaline, septate, 8.0-10.0 µm wide, branched, clamped; **Exoperidial hyphae:** light green in colour, some hyaline (in Congo red), septate, branched, clamp connections present.

Collection examined: Jammu and Kashmir, Poonch, Krishna Ghati, solitary to scattered, humicolous, coniferous forests of *Pinus wallichiana*, Harpreet Kour and Y.P. Sharma, HBJU 231, July 2013.

Edibility: Not eaten in the study area but reported to be edible from other regions (Thakur, 1980).

Distribution: Earlier reported from Western Himalayas, West Bengal (Thakur, 1980).

8. *Suillus granulatus* (L.) Roussel, F. Calvados: 34 (1796)

Synonymy: *Agaricus granulatus* (L.) Lam., *Encycl. Meth. Bot.*1 (1): 51 (1783)
Boletus lactifluus (With.) J. Blum, *Bull. trimest. Soc. Mycol. Fr.*85: 43 (1969)

Pileus: 5.5-8.0 cm wide, convex, mustard yellow (19'.YO-Y.b), on bruising changes colour to brown, surface sticky; **Pores:** present, yellow in colour; **Stipe:** 4.8-5.5 cm long and 20.5-1.5 cm thick, laterally attached, yellow, solid; **Basidiospores:** 7.2-14.4 x 4.0-6.4 µm, a_vL= 10.8, a_vW= 5.2, Q= 1.8-2.25, elliptical, hyaline (in Congo red), yellowish (in 2% KOH), mono- to multiguttulated; **Basidia:** clavate, 17.6-23.2 x 6.4-8.8 µm, hyaline, guttulated; **Pileus cuticle hyphae:** 8.0-14.0 µm wide, septate, hyaline, branched; **Pileus context hyphae:** 10.0-18.0 µm wide, septate, branched, hyaline, guttated; **Pileocystidia**: clavate, 33.6-56.0 x 4.8-6.4 µm. **Stipe hyphae**: 8.0-12.0 µm wide, hyaline, less branched.

Collection examined: Jammu and Kashmir, Poonch, Krishna Ghati, solitary, gregarious, humicolous, Harpreet Kour and Y.P. Sharma, HBJU 234, August 2012

Distribution: Reported from the forests of Kashmir (Beig *et al.,* 2011).

9. *Lactarius deliciosus* (L.) Gray, *Nat. Arr. Brit. Pl.* (London) 1: 624 (1821)

Synonymy: *Agaricus deliciosus* L., *Sp. pl.*2: 1172 (1753)
Galorrheus deliciosus (L.) P. Kumm., *Führ. Pilzk.* (Zerbst): 126 (1871)
Lactifluus deliciosus (L.) Kuntze, *Revis. gen. pl.* (Leipzig) 2: 856 (1891)

Pileus: 1.8-2.8 cm wide, depressed convex in young specimens and infundibuliform at later stages, grenadine pink colour (7.R-O.D), margins inrolled when young; **Gills:** grenadine pink in colour, arcuate, unequal, sub-distant, became greenish later; **Stipe:** 2.0-2.5 cm long and 0.5-1.1 cm wide, equal, smooth, concorolous, solid, geniculate; **Odour:** mushroomy, agreeable; **Basidiospores:** 8.0-11.2 x 7.2-8.0 µm, a_vL= 9.6, a_vW= 7.6, Q= 1.1-1.4, globose to sub-globose, thick walled, forms partial reticulate pattern, uni- to multiguttulated; **Basidia:** 48.0-55.2 x 10.4-13.6 µm, hyaline,

guttulated; **Sterigmata:** 4 in number, 4.8-9.6 µm long, some sterigmata contained guttations; **Pileus hyphae:** 10.0-18.0 µm wide and inflated upto 44.0 µm; **Stipe hyphae:** upto 24.0 µm, septate, branched, hyaline, clamp connections absent.

Collection examined: Jammu and Kashmir, Poonch, Krishna Ghati and Kanuyian, gregarious in *Pinus roxburghii* forests, fasciculate, humicolous, Harpreet Kour and Y.P. Sharma, HBJU 236, September 2012.

Distribution: It has earlier been reported from Sikkim (Butler and Bisby, 1931) Himachal Pradesh, Uttar Pradesh (Saini and Atri, 1982).

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Wetland flora of West Bengal: Lythraceae J. St.-Hilaire

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Abstract

Considering the total area of wide range of wetlands, West Bengal is occupying the fourth position in India. Most of which are known to support very rich plant diversity. The present work recorded a total of 18 species belonging to five genera of Lythraceae J. St.- Hilaire growing in the aquatic or marshland habitat in West Bengal.

Introduction

Lythraceae J. St.-Hilaire is an important family of flowering plants, which is placed under the Myrtales of Malvids of eudicotclade in APG III system of classification (Chase & Reveal, 2009), with about 31 genera and 600 species, widely distributed in tropical regions of the world but relatively less common in temperate regions (Graham *et al.*, 2005). C. B. Clarke (1879) in *The Flora of British India* first time gave an account of Lythraceae of British India. There he included 11 genera viz., *Ammannia* Linnaeus (18 spp.), *Hydrolythrum* Hooker *f.* (1 sp.), *Woodfordia* Salisbury (1 sp.), *Pemphis* Forster (1 sp.), *Lawsonia* Linneaus (1 sp.), *Crypteronia* Blume (3 spp.), *Lagerstroemia* Linneaus (12 spp.), *Duabanga* Hamilton (1 sp.), *Sonneratia* Linnaeus *f.* (4 spp.), *Punica* Linnaeus (1 sp.) and *Axinandra* Thwaites (2 spp.). Cook (1995) reported 7 species of *Ammannia* Linneaus, 2 species of *Nesaea* Kunth and 29 species of *Rotala* Linnaeus from the fresh water bodies in India. Wetland areas are very suitable habitat for different annual or biannual herbaceous species of Lythraceae. The fresh and salt water bodies of West Bengal are quite rich in various species of Lythraceae (Chowdhury, 2009; Chowdhury & Das 2010). Nasker (1990) reported 3 species of *Ammannia* and 3 species of *Rotala* from lower Gangetic plains of West Bengal. Prain (1903) described 9 genera viz., *Ammannia* (*Rotala*) (13 spp. and 2 subsp.), *Hydrolythrum* (1 sp.), *Woodfordia* (1 sp.), *Lawsonia* (1 sp.), *Crypteronia* (1 sp), *Lagerostroemia* (4 spp and 1 sp), *Duabanga* (1 sp), *Sonneratia* (2 spp.) and *Punica* (1 sp.) from the pre-independence undivided Bengal. West Bengal is the bottleneck state of eastern India with unique topographic features. It spreads from the Himalayas in the north to the Bay of Bengal in the south along with the central Gangetic plains and *rarh* and small hills of South western Bengal (Purulia and Bankura districts). The state lies between 85° 50' and 89° 50' E longitude and 21°38' and 27°10' N latitude and spreading over an area of 88,752 km^2, shearing the international border with Bangladesh, Nepal and Bhutan. This state is having very wide range of climatic zones viz., tropical, sub-tropical, temperate and sub-alpine along with fresh and salt water wetlands of various sizes and shapes. These wetlands are known to host quite rich with diversified flora and fauna (Chowdhury and Das, 2009, 2010, 2011, 2013, 2014; Biswas *et al.* 2012; Chowdhury *et al.* 2014).

The present study focused on the species of Lythraceae J. St.-Hilaire which are widely growing in different water bodies of West Bengal. Through extensive survey during last 12 years accumulated rich and useful information about these plants from Bengal wetlands. The collected minor taxa of Lythraceae of West Bengal wetlands are enumerated below along with their identification keys, description, distribution and uses.

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ENUMERATION

Key to the Genera:

1a. Aquatic floating herbs; fruits 1- seeded and with 2-4 spines.................................. *Trapa*
1b. Marshy or halophytic rooted herbs or trees; fruits with more than one seed, unarmed.. 2

2a. Halophytic trees; calyx thickly coriaceous.. ....*Sonneratia*
2b. Hydrophytic herbs; calyx thin, membranous 3
3a. Petals brick-red or absent; capsules open with lateral slit or irregularly at base ....*Nesaea*
3b. Petals pink – purple or absent; capsules open with longitudinal slit or irregulary..... 4
4a. Flowers in axillary cymes ....*Ammannia*
4b. Flowers solitary or in terminal spikes... .... *Rotala*

AMMANNIA Linnaeus, Sp. Pl. 1: 119. 1753.

Key to the species:

1a. Styles up to 0.5 mm long..............................2
1a. Styles 1 – 5 mm long 3
2a. Lamina and sepals pubescent; sepal appendage distinct................ *A. verticillata*
2b. Lamina and sepals glabrous; sepal appendage absent.................... *A. baccifera*
3a. Capsules 2 – 3.6 mm in diameter; sepal lobes erect in capsule............. *A. multiflora*
3b. Capsules upto 1.5 mm in diameter; sepal lobes reflexed in capsule .... *A. verticillata*

Ammannia auriculata Willdenow, Enum. P1. Hort. Berol. 1:7,t.7. 1803. Cook, Aqua. Wetl. Pl. Ind. 249.1995.

Hyperhydrate or tenagophyte; erect or decumbent, 10-40 cm tall. Lamina linear-lanceolate, auriculate. Cymes pedunculate. Hypanthium 1.5-3 mm long, vertically 8-10 green ribbed; ribs obscure on fruits. Epicalyx minute. Petals obovate-cuneate or absent; stamens inserted above the middle of the hypanthium; ovary 1-2mm long. Capsule slightly exceeding the hypanthium. Seeds discoid.
Flowers & Fruits: August - November.
Distribution: Tropical and warm temperate regions of World; common throughout the Bengal-plains.

Ammannia baccifera Linnaeus, Sp. Pl. 120. 1753; Clarke in Hooker *f.*, Fl. Brit. Ind. 2: 569. 1879; Prain, Beng. Pl. 2: 500. 1903; Cook, Aqua. Wetl. Pl. Ind. 249.1995.(Plate I: F)

Hyperhydrate or tenagophyte; annual, erect, glabrous herbs. Stem reddish, quadrangular. Leaves opposite decussate; lamina sub- sessile, oblong and narrowly elliptic. Inflorescence axillary, sessile cluster. Flowers pink, bisexual. Capsules globose, irregularly dehiscing, depressed with black seeds.
Flowers & Fruits: July – March.
Distribution: Tropical and warmer regions of Africa, Asia and Australia; abundant throughout the Bengal-plains.
Uses: Leaves used to clear blisters and also given against rheumatism and fever (Misra & Dash, 2002; Pandey *et al.*, 2002).

Ammannia multiflora Roxburgh, Fl. Ind. 1: 447. 1820; Clarke in Hooker *f.*, Fl. Brit. Ind. 570. 1879; Prain, Beng. Pl. 1: 500. 1903; Cook, Aqua.Wetl. Pl. Ind. 250. 1995 (Plate I: B).

Hyperhydrate or tenagophyte; erect branched herbs. Stem quadrangular, hard. Lamina elliptic, sessile. Flowers reddish, axillary, cymes peduncled, compound. Capsules small, globose with persistent style.
Flowers & Fruits: November – February.
Distribution: Tropical and warmer regions of the old World; abundant throughout the plains.

Ammannia verticillata Lamarck, Encycl.[J. Lamarck &*al.*]1(1): 131. 1783; Cook, Aqua.Wetl. Pl. Ind. 251.1995. *Ammannia salicifolia* Heirn in Oliver, Fl. Trop. Afr. 2: 278. 1871. excl. syn.; Clarke in Hooker *f.* Fl. Brit. Ind. 2: 569. 1879; Prain, Beng. Pl.1: 501. 1903.

Hyperhydrate or tenagophyte; erect herbs; stem quadrangular, hard, purple, terete. Leaves opposite decussate; lamina lanceolate, rounded at the base. Inflorescence clusters many flowered, sessile; calyx not covered by calyx teeth. Fruits globose, irregularly dehiscing.
Flowers & Fruits: October – January.
Distribution: Pantropical; abundant throughout the Bengal-plains.

NESAEA Kunth, Nova Genera *et* Sp. Pl. 6 (ed. folio). 1823.

Key to the species:
1a. Lamina base cordate and sub-amplexicauled; petals absent......*N. brevipes*

Plate- I. Lythraceae from wetlands: A. *Rotala rotundifolia* (Buchanan-Hamilton) Koehne B. *Ammannia multiflora* Roxburgh C. *Sonneratia caseolaris* (Linnaeus) Engler D. *Trapaincisa* Siebold & Zuccarini E. *Trapanatans* Linnaeus F. *Ammannia baccifera* Linnaeus G. *Nesaea brevipes* Kiehne

1b. Lamina base attenuate; petals present ....*N. prostrata*

Nesaea brevipes Koehne, Bot. Jahrb .S yst. 3: 326. 1882. *Ammannia cordata* Wight & Arnott,

Prodr. 304. 1834, non Hiern; Prain, Beng. Pl. 1: 501. 1903; Cook, Aqua. Wetl. Pl. Ind. 252. 1995 (Plate I: G).

Hyperhydrate or tenagophyte; annual, erect or diffused, glabrous herbs. Leaves opposite, oblong – cordate.Flowers 2 – 6, pedicel short; calyx lobes 4, campanulate, green. Capsules globose with sub hemispheric seeds.

Flowers & Fruits: November – March.

Distribution: Indian sub-continent; abundant throughout the Bengal-plains.

Nesaea prostrata (Buchanan-Hamilton ex Dillwyn) Suresh in D.H. Nicolson, C. R. Suresh & K.S. Manilal, Interpret.Van Rheede's Hort. Malab. 168. 1988; Cook, Aqua. Wetl. Pl. Ind. 252. 1995. *Ammannia prostrata* Buchanan-Hamilton *ex* Dillwyn, Rev. Hortus Malab. 40. 1839; D.J. Mabberley in Taxon 26(5–6): 533. 1977, as *'Ammania'.* 1839.

Hyperhydrate or tenagophyte; annual, erect or decumbent herbs. Lamina linear-lanceolate to oblong. Cyme sessile. Flowers sessile; bracteoles lanceolate to oblong; sepals 4-5; petals 4-5, pink, rarely absent; stamens 4-5. Capsules globose.

Flowers & Fruits: November – March.

Distribution: India, Sri Lanka and Australia; throughout the Bengal-plains; rare.

ROTALA Linnaeus, Mant. Pl. Altera 143. 1771.

Key to the species:

1a. Leaves whorled..2
1b. Leaves decussate..3
2a. Racemes terminal; petals present; stamens 4.. *R. wallichii*
2b. Flowers axillary solitary; petals absent; stamens 1-4 ....*R. mexicana*
3a. Flowers in terminal spikes; stigma massive, discoid................................*R. rotundifolia*
3b. Flowers in axillary spikes; stigma capitate to punctiform..4
4a. Lamina margin translucent to opaque white cartilaginous; capsules 2-valved ...*R. indica*
4b. Lamina margin green, membranous; capsules 3- or 4-valved.............................5
5a. Stems broadly 4-winged; bracts smaller than foliage leaves; flowers in axillary spikes or sub-sessile on main stem............................6
5b. Stems terete or 4-angled; bracts like foliage leaves; flowers sessile on main stem..*R. rosea*
6a. Epicalyx segments between sepals absent; sepals 4.................................. *R. cordata*
6b. Epicalyx segments between sepals setiform, about half of corolla tube; sepals 5..*R. densiflora*

Rotala cordata Koehne, Bot. Jahrb. Syst. 1: 172. 1880. Cook, Aqua. Wetl. Pl. Ind. 255.1995. *Rotala diversifolia* Koehne, Bot. Jahrb. Syst. 41(2): 77. 1907. *Ammannia cordata* Wight & Arnott, Prodr. Fl. Ind. Orient. 1: 304. 1834. Prain, Beng. Pl. 1: 501. 1903. Clarke in Hooker *f.,* Fl. Brit. Ind. 2: 570.1879.

Hyperhydrate or tenagophyte; annual Herbs. Stem branched, 4-winged. Leaves decussate, narrowly oblong to lanceolate. Bracts lanceolate to oblong. Flowers solitary, subsessile, in bracts of axillary spikes; bracteoles minute, at base of floral tube, scarious. Floral tube 4-merous, broadly campanulate; sepals 4, pink-tinged; petals 4, obovate; stamens 4; ovary globose; style exserted. Capsules globose.

Flowers & Fruits: November – March.

Distribution: NE India, China, Laos, Thailand, Vietnam; throughout the Terai of Darjeeling and Jalpaiguri districts, ascending upto 400 m; less common.

Rotala densiflora (Roth) Koehne, Bot. Jahrd.1: 164. 1880; Datta & Majumdar, Bull. Bot. Soc. Beng. 20 (2): 89. 1966. *Ammannia densiflora* Roth, R. & S. Syst. Veg. 3: 394. 1818. *Ammannia pentandra* Roxburgh, Fl. Ind. 1: 488. 1820; Prain, Beng. Pl. 1: 500. 1903.

Hyperhydrate or tenagophyte; spreading herbs with divarcating floriferous branches, fleshy pink. Leaves elliptic oblong. Flowers small, bracteate, bracteoles scarious, axillary, solitary; epicalyx segments present between sepals, setiform; petals 5, bright pink or white, equal to or surpassing sepals, persistent. Stamens 5. Capsules 3 – 4 valved. Seeds black.

Flowers & Fruits: August – February.

Distribution: Northern parts of India to Australia; common throughout the Bengal-plains.

Rotala indica (Willdenow) Koehne, Bot. Jahrb. Syst. 1: 172. 1880. Cook, Aqua. Wetl. Pl. Ind. 257.1995. *Peplisindica* Willdenow, Sp. Pl. 2: 244. 1799.

Hyperhydrate or tenagophyte; ascending or erect, annual Herbs. Leaves decussate, lamina obovate-elliptic or obovate-oblong, margin translucent to opaque. Bracts leafy or distinctly smaller on axillary spikes.Flowers in axillary spikes or sessile in bracts on main stem; bracteoles linear. Floral tube 4-merous; sepals 4, lanceolate-deltoid; petals 4, pink; stamens 4; ovary ellipsoid; style as long as ovary.Capsules ellipsoid.

Flowers & Fruits: September – April.

Distribution: S & SE Asia; introduced to Africa, Europe and N America; common throughout the Bengal-plains.

Rotala mexicana Chamisso & Schlechtendal, Linnaea 5: 567. 1830. Grierson & Long, Fl. Bhut. 2 (1): 274. 1991. *Ammannia pygmaea* Kurz, J. Bot. 5: 376. 1867; Prain, Beng. Pl. 1: 500. 1903.

Hyperhydrate or tenagophyte; annual, floating, erect or ascending, minute herbs. Leaves whorled, aerial leaves narrowly lanceolate to broadly. Flowers sessile, solitary, axillary; bracteoles linear; petals absent; stamens (1 or) 2 or 3 (or 4), included; ovary subglobose. Fruits capsule.

Flowers & Fruits: September – January.

Distribution: Tropical and warmer regions of world except Pacific Islands, NE Africa, Arabia; throughout the plains North Bengal; rare.

Rotala rosea (Poiret) C.D.K. Cook in Boissiera 29: 86. 1979; Panda & Das, Fl. Sambalp. 150. 2004. *Ammannia rosea* Poiret in Lamarck, Encycl. Meth. Bot. (Suppl. 1) 329. 1810. *A. pentandra* Roxburgh, Fl. Ind. (ed. 1) 1: 448. 1820; Clarke in Hooker *f.*, Fl. Brit. Ind. 2: 568. 1879; Prain, Beng. Pl. 1: 500. 1903.

Hyperhydrate or tenagophyte; annual, terrestrial or amphibious, ascending herbs. Leaves opposite decussate; lamina linear – lanceolate to lanceolate – oblong. Flowers solitary, sessile, axillary; bracteoles linear. Floral tube campanulate; epicalyx segments setiform, equaling sepals; petals 5; stamens 5; anthers reaching margin of floral tube; ovary globose. Capsules globose.

Flowers & Fruits: August – September.

Distribution: S & SE Asia; common throughout the Bengal-plains.

Rotala rotundifolia (Buchanan-Hamilton) Koehne in Bot. Jahrb.1: 175.1881; Bora & Kumar, Flor. Div. Ass. 158. 2003. *Ammannia rotundifolia* Buchanan–Hamilton in Don Prodr. 220. 1825; Clarke in Hooker *f.*, Fl. Brit. Ind. 2: 566. 1828; Prain, Beng. Pl. 1: 500. 1903.(Plate I: A)

Hyperhydrate or tenagophyte; extensively creeping and rooting herbs with red stem. Lamina sessile, orbicular or broadly elliptic – rounded. Flowers pinkish sessile, closely packed in terminal simple or panicle spikes; calyx tube campanulate, sepals 4; petals 4, pink; stamens 4; ovary pyriform to globose. Capsules 4-valved ellipsoid; seeds elliptic peltate.

Flowers & Fruits: November – April.

Distribution: S & SE Asia; abundant throughout the Bengal-plains.

Rotala wallichii (Hooker *f.*) Koehne, Bot. Jahrb. Syst. 1: 154. 1880. Cook, Aqua. Wetl. Pl. Ind. 262.1995. *Hydrolythrum wallichii* Hooker *f.* in Bentham & Hooker *f.*, Gen. Pl. 1: 777. 1867.

Hyperhydrate or tenagophyte; emergent, perennial, Herbs. Leaves whorled; aerial leaves 3-12 whorled, linear to oblong, submerged leaves filiform. Bracts much reduced in inflorescence, oblong or ovate. Flowers 5-8-whorled per node, shortly pedicellate in a bracteate raceme; bracteoles short. Floral tube 4-merous, campanulate; sepals 4; epicalyx absent; petals 4, light red or pink, orbicular; stamens 4; ovary globose; style included. Capsules globose,

Flowers & Fruits: September – April.

Distribution: S & SE Asia; common throughout the Bengal-plains.

Sonneratia Linnaeus *f.*, Suppl. Pl. 38, 252.1782; *nom. cons.*

Key to the species:

1a. Lamina elliptic-lanceolate; stigma peltate.. *S. apetala*

1b. Lamina ovate-oblong; stigma capitate..2

2a. Corolla absent; ovary 6-celled...*S. griffithii*

2b. Corolla red; ovary 16-20 celled...*S. caseolaris*

Sonneratia apetala Buchanan-Hamilton, Syems. Embassy Ave. 3: 477. 1800; Prain, Beng. Pl. 1: 505. 1903; Naskar, Pl. Wealth Ganga Delta 1: 351. 1993.'***Tak Keora'***

Intertidal; evergreen tree with erect pneumatophores. Leaves simple, opposite; lamina elliptic, obovate. Inflorescence axillary, 3-flowered cyme. Flowers white; calyx 4 lobed, reflex; petals absent, stamens numerous, filament bent inwards in bud; ovary 2-20 celled, stigma large. Fruit a berry.

Flowers & Fruits: December – July.

Distribution: Native to Bangladesh, India, Myanmar, Sri Lanka, China; inter-tidal river flats of mangrove swamps, muddy flats of Sundarbans; common.

Sonneratia caseolaris (Linnaeus) Engler, Engler & Prantle, Nachtr. 261. 1897; Naskar, Pl. Wealth Ganga Delta 1: 351. 1993. *Rhizophora caseolaris* Linnaeus, Herb. Amboin. (Linnaeus) 13. 1754. '***Chakkeora'*** (Plate I: C)

Intertidal; evergreen tree, glabrous throughout. Pneumatophore present. Leaves opposite; lamina elliptical, oblong or ovate, entire, leathery. Flowers 1-3 at end of drooping twigs malodorous, nocturnal; hypanthium with 6-8 calyx lobes; petals dark or blood-red; stamens numerous, with threadlike filaments; pistil with 16-21 celled; style long, stout. Fruit berry.

Flowers & Fruits: March – October.

Distribution: SE Asia, Philippines, N Australia; common in inter-tidal river flats and inner estuary of mangrove swamps of Sundarbans.

Sonneratia griffithi Kurz, J. Asiat. Soc. Bengal 40 (2): 56. 1871; Naskar, Pl. Wealth Ganga Delta 1: 351. 1993. *Sonneratia acida* Linnaeus; Prain, Beng. Pl. 1: 505. 1903.'***Ora'***

Intertidal; evergreen tree with numerous pneumatophores. Leaves simple, opposite decussate, extipulare; lamina obovate. Inflorescence solitary cyme. Flowers pedicellate; sepals 6 – 8; petals absent; stamens numerous, free; carpels 6, style 1. Fruits berry.

Flowers & Fruits: April – October.

Distribution: Africa, Indo-Malaysia, Australia; common in inter-tidal river flats and outer estuary of mangrove swamps of Sundarbans.

Trapa Linnaeus, Sp. Pl. 1: 120. 1753.

Key to the species

1a. Lamina much villose beneath; margin incised; fruits with 2 soft spines..*T. natans*

1b. Lamina slightly villose beneath; margin not incised; fruit with 4 stiff spines.......*T. incisa*

Trapa incisa Siebold & Zuccarini, Abh.Math.-Phys. Cl. Königl. Bayer. Akad. Wiss. 4(2): 134. 1845. *Trapa natans* var.*incisa* Makino, Bot. Mag. (Tokyo) 1: 105. 1887 – 1892. *Trapa bispinosa* var. *incisa* (Siebold & Zuccarini) Franchet & Savatier, Nakai, Fl. Kor. 2: 490. 1911. Clarke in Hooker *f.*, Fl. Brit. Ind. 2: 590. 1879; Prain, Beng. Pl. 1: 508. 1903. (Plate I: D)

Epihydrate; floating herbs. Floating lamina in rosettes, rhombic – triangular, glabrous or sparsely pubescent on veins, margin coarsely and sharply incised-dentate distally. Petals pink to pale purplish or white. Fruit narrowly rhombic, 4-horned, surface variously ribbed to smooth, crest absent; horns conic.

Flowers & Fruits: May – November.

Distribution: S & SE Asia, China, Japan, Korea, Laos, Malaysia and Vietnam; abundant in stagnant water bodies throughout the Bengal-plains.

Uses: Fleshy larger cotyledon is edible.

Trapa natans Linnaeus, Sp. Pl. 1: 120. 1753. *Trapa natans* Linnaeus var. *bispinosa* (Roxburgh) Makino in Iinuma, Sumoku-Dzusetd "ed. 3", 1: 137.1907; Prain, Beng. Pl.1: 508. 1903. *Trapa bispinosa* Roxburgh, Pl. Cor. 3: t. 234. 1815; Clarke in Hooker *f.*, Fl. Brit. Ind. 2: 590. 1879. '***Singara Phal'*** or ***'Pani Phal'*** (Plate I: E)

Epihydrate; floating herbs. Floating leaves in rosettes, rhomboid, crowded in the upper part of stem; submerged ones dissected. Flowers

solitary, axillary; calyx lanceolate, acute; corolla white; pubescent; stamens 4. Nuts 3-angled with 2 spiny horns.
Flowers & Fruits: September – January.
Distribution: Tropical parts of Asia; throughout the Bengal plains; commonly cultivated and marketed in large amount.
Uses: Fleshy larger cotyledon is edible.

Discussion

Lythraceae is a family of both terrestrial and wetland elements. 18 species of wetland plants recorded from West Bengal during the present survey and are representing five genera except three tree species of *Sonneratia* that are growing in salt water marshes of Sunderban mangroves, all others are small herbs growing in various depths, mostly as emerge plants near the periphery of water bodies. Majority of the recorded species are widely distributed in the tropical wetlands of the state except *Nesaea prostrata* and *Rotala mexicana*, which are quite rare in distribution and are only recorded from few wetlands of South and North Bengal respectively. Species of *Rotala* and *Neasea* are decumbent fleshy herbs growing at the peripheral marshy zone of different wetlands forming a dense mat along with several species of grasses, sedges and sometimes with isolated *Ammannia* sp.

The occurrence of these species appears to be normal but, considering broadly, the abundant use of herbicides in fields of wetland crops greatly affecting their population in such habitat. Most part of the population of *Sonneratia* spp. is growing near the estuaries but legal and illegal clearing activities are, certainly, affecting its population.

Like any other hydrophyte and/or wetland plants these are also prone to be affected due to different changed conditions in the habitat. If the wetlands are conserved properly then, automatically, most of these species will continue to survive in their natural habitat.

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Morphological characterization of rice cultivars their root colonization with arbuscular mycorrhizal fungi and screening for field resistance caused by brown spot disease

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Abstract

Variability in seed morphology was studied in 15 rice cultivars using qualitative and quantitative characters. Germplasm of these indigenous rice landraces were collected from Bijanbari, Kalimpong, Sikkim, Malda, Siliguri and UBKV (Uttar Banga Krishi Viswavidyalaya). Data were recorded for traits such as Kernel colour, Seed coat colour, Aroma, Presence of Awn and Length of the seed. A total of 9 landraces had white kernel colour while 4 had brown and 2 had greyed-orange .The seed coat colour variation in different landraces ranged from Golden yellow, Yellow, Red and Black. 6 landraces were having aroma whereas 9 had no aroma and lastly 11 landraces were found to have awn and 4 were awnless.UBKV-4 was longest in length with 1.1 cm and Sano masuri being the smallest of 0.4 cm. Establishment of disease in naturally infected rice cultivars were observed and disease index was calculated. Arbuscular Mycorrhizal Fungi (AMF) were screened from rhizosphere of fifteen rice cultivars grown on experimental field using wet sieving and decanting method. Microscopical observation revealed the presence of different genus of AM fungi present in the roots as hyphae, spores and sporocarp. Among the different AM fungi species of *Glomus* sp.were found to be high in all the fifteen cultivars of rice plants followed by *Gigaspora*, *Scutellospora* and *Acaulospora*. Histopathological study of roots showed the presence of vesicles and arbuscules. AMF infection and total number of spores per 100 grams of soil were recorded. Present study evaluates the study of different AMF population and their histopathology harbouring in the rhizosphere of rice.

Keywords: Rice cultivars, Morphological traits, AM Fungi.

Introduction

Rice (*Oryza sativa* L., family Poaceae) is the leading staple food crop of India, grown in almost all the states, covering more than 30 per cent of the total cultivated area (Adhikari *et al.*, 2012; Chakravorty *et al.*, 2013). West Bengal is called as 'bowl of rice' with over 450 rice landraces (Deb D., 2005; Chatterjee *et al.*, 2008). Diversity studies in rice using morphological characters were done on improved and ancestral rice varieties of Philippines (Caldo *et al.*, 1997; Juliano *et al.*, 1998) and on Asian wild cultivated indigenous rice in Yunnan, China (Zeng *et al.*, 2003).

Agro-morphological traits, both qualitative and quantitative have been commonly and traditionally used to estimate relationships between genotypes (Goodman M.M., 1972). Variation due to adaptation to specific ecosystems selection and socio-economic condition resulted in differentiation in different named landraces of a region (Bajracharya *et al.*, 2006). Variability study for rice landraces from West Bengal was undertaken by (Chakravorty *et al.*, 2013). Keeping in view the under representation of rice landrace diversity from West Bengal and Sikkim 15 different rice cultivars were selected and studied for seed morphology, associated knowledge on local use of collected landraces was recorded to help in characterization of rice germplasm from this region.

Arbuscular Mycorrhizal Fungi (AMF) are vital components of the microbial soil community forming the most commonly occurring underground symbiosis between members of phylum *Glomeromycota* and roots of 80% of all terrestrial plant species (Wang *et al.*, 2008; Schüßler *et al.*, 2001). AMF are the key species groups that inter-connect plants into a functional web (Hegalson *et al.*, 1998), extending plant root systems and thereby, facilitates plants uptake of soil nutrients of poor mobility, especially phosphorus (Smith and Read, 2008).

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Besides, AMF improve plant fitness by improving seedling establishment, plant fecundity, tolerance to some root pathogens, water relations and formation and stability of soil aggregates (Read, 1999; Newsham *et al.*, 1995a). Efforts are being undertaken to develop a bio formulation which can reduce the disease occurrence.

Considering the importance of AMF for disease resistance in rice plants present investigation was made to assess the AMF population from fifteen different rice varieties collected from different areas of hilly and plain regions and were grown on experimental field of Immuno Phytopathology Lab, Department of Botany, NBU.

Materials and methods

Collection of Rice Seeds

Rice seeds were collected from different regions of West Bengal and Sikkim. Brimful, Champasari and Black Nuniya from Bijanbari. Kaberi 9090, Loknath 505 and Gouraknath 509 from Siliguri. Sano masuri and Adde from Sikkim. Attheu and Maiti from Kalimpong .Swarnamasuri and Tulai panji from Malda and finally UBKV-1, UBKV-4 and UBKV-5 from Uttar Bangha Krishi Viswavidyalaya (UBKV) respectively.

Morphological study and measurement of seeds

Seed morphology was recorded paying attention to Kernel colour, seed coat colour, aroma, presence of awn and finally the length of the seed was noted.

Disease Assessment

Establishment of naturally occurring brown spot disease was observed and disease severity was assessed in terms of lesion number per leaf and infection index calculated as described by Adlakha et al. (1984). For percent disease index (PDI) calculation, the following formula was used-[(class rating x class frequency)/(total no. of leaves x maximum rating)] x 100.

Isolation of AMF spores from soil

Arbuscular mycorrhizal fungal spores were screened from soil samples of fifteen rice varieties rhizosphere by the wet sieving and decanting method (Gerdeman & Nicholson, 1963). Soil samples (100gm each of the root zone) were collected, suspended in water (1 lt) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50 µm) arranged one below the other. The spores were picked by the help of bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one litre of boiled distilled water) at 4°C for further study and observation. It is evident from various studies that each plant has multiple AM fungi population.

Identification of AMF spores

Spore samples were separated according to their morphology size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified with the help of standard keys (Walker, 1981; Schneck and Perez, 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layer, position and abundance), hyphal branching patterns, the diameter, structure (specially near entry points) and the staining intensity of hyphae.

Spore count

Rhizosphere soil (100gm) was taken and suspended in 250ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores was then counted and spore percentage of different genera was obtained.

Histo-pathological analysis

The root specimen were taken from field and washed with tap water. The roots were cut into pieces, after washing treated with 10% KOH added, kept in water bath for 1hr, then 1% HCl was added to neutralize the alkalinity. The root pieces were then washed with water (after 30 min) and staining was done by shimmering the roots in cotton blue: lactophenol (1:4) for 3-4 min with mild heating. Degree of contrast between fungal tissues and back ground plant cell was obtained according to the duration of storage of tissues. 1% HCl was added to acidify the tissues, as most histological stains are acidic. A little amendment in this process is noteworthy because it has been noticed that extraradical spore bearing hyphae and other extraradical fungal tissue with root segments are destroyed or dissolved when it is boiled in hot water bath at 90°C twice with 2% KOH followed by 0.05 cotton blue and lacto glycerol for staining the internal structures of AMF inside the root segment i.e. arbuscules, vesicles, auxilliary cells etc. The total staining process can be done without heating but keeping the root fragments in 1-2% KOH for 24-48 hrs in a Petri dish and another 2-18 hrs in cotton blue and lactoglycerol with minimum movement of the samples yields remarkable results. In this method the spore bearing hyphal structures, auxiliary cells etc. are clearly visible and percent colonization can be determined with better accuracy. After preparing the roots the hyphal structures were viewed under dissecting stereo-microscope under 20X and 40X magnification.

Root colonization

Percent root colonization was estimated by using slide method by (Giovannetti and Mosse, 1980). All the infected and uninfected segments of root tissue and the percentage of infection was calculated as follows
AMF infection (%)=[infected root segments/total fragments of root taken] X 100.

Results and Discussion

Fifteen different rice cultivars were collected from different regions of West Bengal and Sikkim the cultivar name, type, origin and its GPS Location is given in (Table 1).

Table 1. Rice cultivars and its localization.

Sl.No.	Rice Cultivars	Cultivar type	Origin	GPS Location
1.	Brimful	Ethnic	Bijanbari	27° 02′N 88° 07′E/ 27.04° N 88
2.	Champasari	Ethnic	Bijanbari	27° 02′N 88° 07′E/ 27.04° N 88
3.	Black Nuniya	Local	Bijanbari	27° 02′N 88° 07′E/ 27.04° N 88
4.	Kaberi 9090	Commercial	Siliguri	26.7100° N,88.4300°E
5.	Loknath 505	Commercial	Siliguri	26.7100° N,88.4300°E
6.	Gouraknath 507	Commercial	Siliguri	26.7100° N,88.4300°E
7.	Sano Musuri	Ethnic	Sikkim	27.3300° N,88.6200°E
8.	Adde	Ethnic	Sikkim	27.3300° N,88.6200°E
9.	Attheu	Ethnic	Kalimpong	27° 04′N 88° 28′E/27.06,88.47°
10.	Maiti	Ethnic	Kalimpong	27° 04′N 88° 28′E/27.06,88.47°
11.	Swarnamasuri	Local	Malda	25.0000° N,88.1500°E
12.	Tulaipanji	Local	Malda	25.0000° N,88.1500°E
13.	UBKV-1	Research	UBKV	26° 24′15″ N, 89° 23′5″E
14.	UBKV-4	Research	UBKV	26° 24′15″ N, 89° 23′5″E
15.	UBKV-5	Research	UBKV	26° 24′15″ N, 89° 23′5″E

Fig. 1.Fifteen different rice cultivars.

Table 2: Morphological diversity of rice cultivars.

Sl. No.	Rice Cultivar	Area of collection	Kernel colour	Seed coat colour	Aroma	Presense of Awn	Length of the seed (cm)
1.	Brimful	Bijanbari	Brown	Red	Present	Absent	0.9
2.	Champasari	Bijanbari	White	Red	Absent	Present	0.8
3.	Black Nuniya	Bijanbari	Brown	Black	Present	Absent	0.7
4.	Attheu	Kalimpong	White	Yellow	Present	Absent	0.9
5.	Sano Masuri	Sikkim	White	Yellow	Absent	Absent	0.4
6.	Loknath 505	Siliguri	White	Golden Yellow	Absent	Absent	0.8
7.	Gouraknath 509	Siliguri	White	Golden Yellow	Present	Absent	0.7
8.	Kaberi 9090	Siliguri	White	Golden Yellow	Absent	Absent	0.9
9.	Adde	Sikkim	Brown	Yellow	Present	Absent	0.5
10.	Maiti	Kalimpong	Brown	Yellow	Absent	Absent	0.6
11.	Swarnamasuri	Malda	Greyed orange	Red	Absent	Absent	0.7
12.	Tulaipanji	Malda	Greyed orange	Golden Yellow	Present	Present	0.7
13.	UBKV-1	UBKV	White	Yellow	Absent	Present	0.9
14.	UBKV-4	UBKV	White	Red	Absent	Present	1.1
15.	UBKV-5	UBKV	White	Yellow	Absent	Absent	1.0

Table 3: Population count of AM Fungi in rhizosphere of fifteen different rice cultivars and percentage colonization in root

Sl. No.	Rice Cultivars	Percentage of VAM spore in soil (%)					Root colonization (%)
		Glomus	*Gigaspora*	*Scutellospora*	*Acaulospora*	*Entrophospora*	
1	Loknath 505	78.04	19.59	1.68	0.33	0.33	99 %
2	Gouraknath 509	83.85	15.09	-	1.04	-	91 %
3	Kaberi 9090	67.17	27.30	0.61	4.90	-	93%
4	Champasari	80.0	20	-	-	-	90%
5	Brimful	85.52	13.15	1.3	-	-	99%
6	Black Nuniya	83.33	13.88	-	2.77	-	94%
7	Adde	66.66	31.81	-	1.51	-	95%
8	Sano Masuri	65.94	33.74	-	.30	-	93%
9	Maiti	83.30	13.88	-	2.77	-	97%
10	Attheu	78.02	17.48	1.1	3.36	-	96%
11	Swarnamasuri	69.7	23.25	5.81	1.16	-	93%
12	Tulai Panji	65.19	33.77	1.04	-	-	95%
13	UBKV-1	90.39	5.64	1.12	2.82	-	98%
14	UBKV-4	60.30	36.43	0.75	2.51	-	100%
15	UBKV-5	50.07	41.07	2.52	6.31	-	98%

Seed Morphological Diversity of all the cultivar was observed (Fig. 1) and was seen that a total of 9 landraces had white kernel colour while 4 had brown and 2 had greyed-orange .The seed coat colour variation in different landraces ranged from Golden yellow, Yellow, Red and Black. 6 landraces were having aroma whereas 9 had no aroma and lastly 11 landraces were found to have awn and 4 were awnless. UBKV-4 was longest in length with 1.1 cm and Sano masuri being the smallest of 0.4 cm as shown in (Table 2). Table 3 shows the percentage of different AM fungi in the each soil samples and the maximum population was found to be of *Glomus* sp. followed by *Gigapora* sp., *Acaulospora* sp., *Scutellospora* sp. and

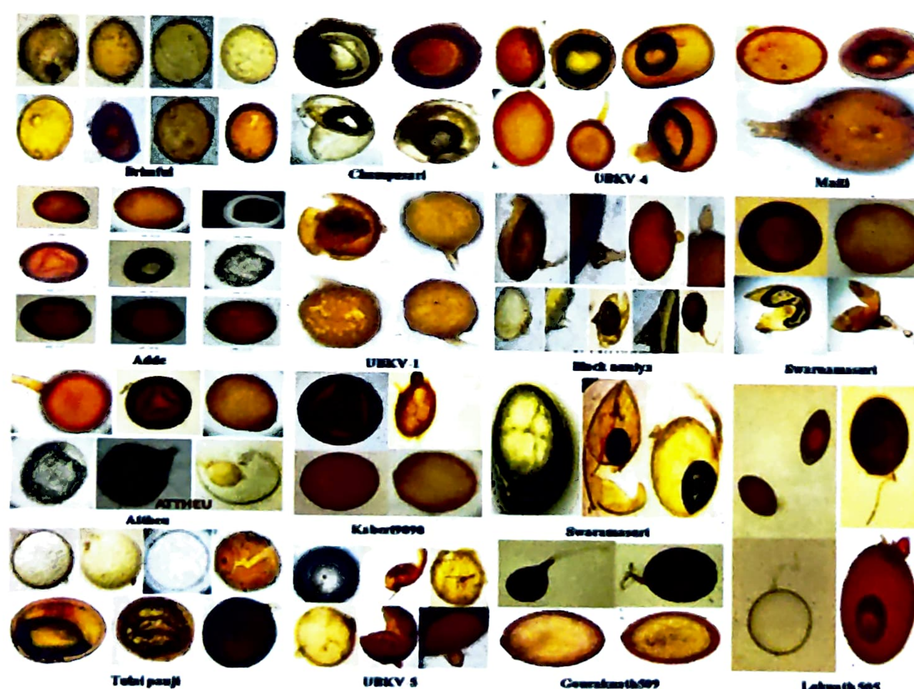

Fig. 2 AMF population collected from rhizospheric soils of rice

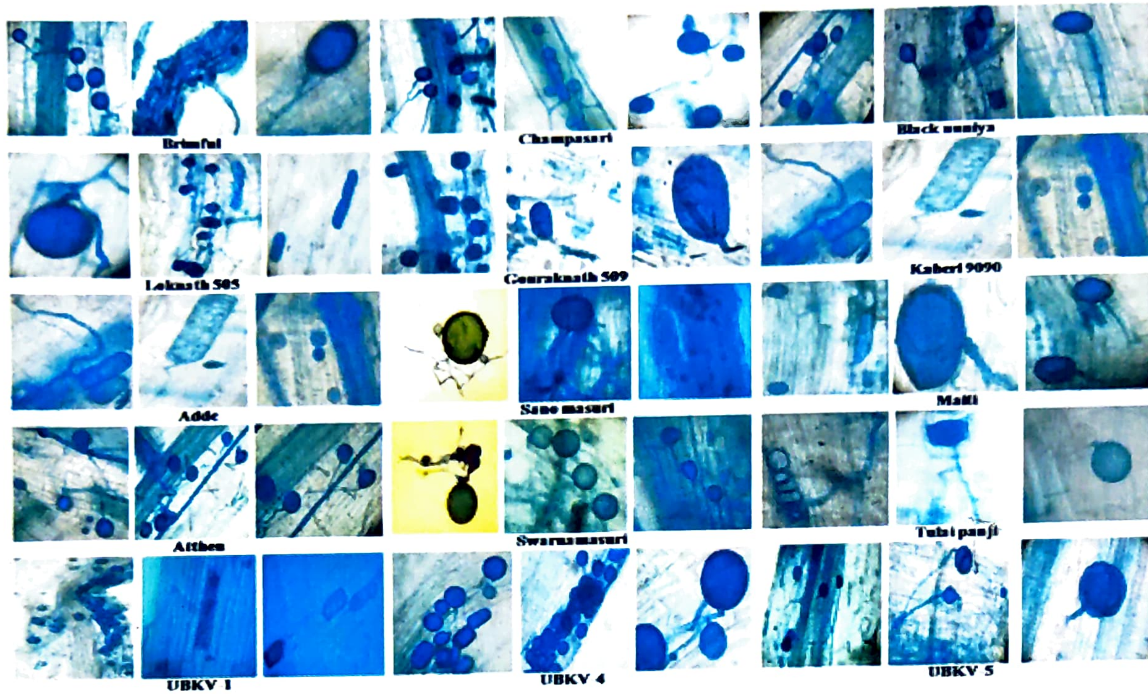

Fig. 3: Observation of rice root colonisation by AMF.

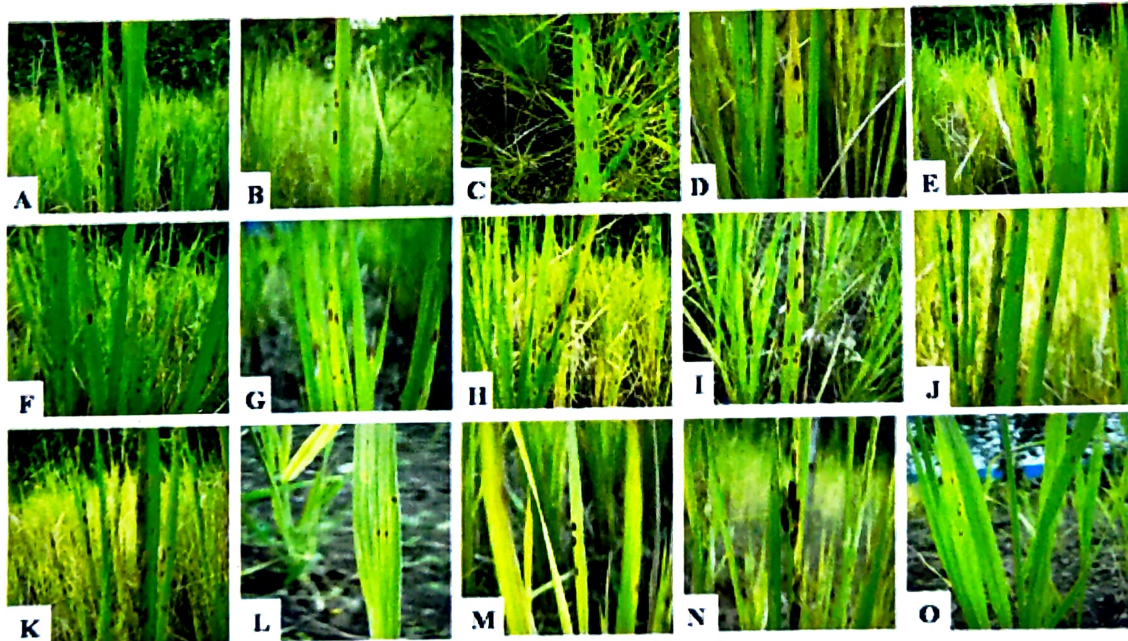

Fig. 4: Rice cultivars grown in experimental plots for study of AMF population and root colonisation. (A) Brimful,(B) Champasari, (C) Black nuniya, (D) Loknath 505, (E) Gouraknath 509,(F) Kaberi 9090 (G) Adde, (H) Sano masuri, (I) Maiti, (J) Attheu, (K) Swarnamasuri, (L) Tulai panji, (M) UBKV-1,(N) UBKV-4,(O)UBKV-5.

Enthropospora sp. was found in the rhizosphere of one of the cultivars.

Morphological and topographical characteristics of plant organs such as the shape and size of seeds and the structure of incidental

features have been useful weapons in identifying and classifying the plant and weed species (Noda *et al.*, 1985). Awn less seed is an improved trait and high diversity in seed shapes and pericarp color may be important for developing quality rice to meet diverse consumer demand.

Table 4: Disease index showing the establishment of natural disease.

Sl.No	Rice cultivars	Disease index (PDI %)
1.	Brimful	62.28
2.	Champasari	51.76
3.	Black Nuniya	52.72
4.	Kaberi 9090	58.47
5.	Loknath 505	41.66
6.	Gouraknath 507	50.05
7.	Sano Musuri	48.36
8.	Adde	59.82
9.	Attheu	49.44
10.	Maiti	47.89
11.	Swarnamasuri	53.33
12.	Tulaipanji	51.85
13.	UBKV-1	41.62
14.	UBKV-4	43.78
15.	UBKV-5	43.66

The role of below-ground soil organisms interacting with plant roots has gained increased attention in recent years (e.g. Reynolds et al., 2003; van der Putten, 2003; Callaway et al., 2004), and the interactions between beneficial and pathogenic organisms have been identified as being particularly relevant due to their important implications for plant fitness (e.g. Schippers et al., 1987; Fitter and Garbaye, 1994; Bever, 2003). Arbuscular Mycorrhizal Fungi were collected and screened from the rhizospheric soil of fifteen rice cultivars grown on experimental plots. The different types of spores which were observed in the rhizosphere of rice soil have been identified. On observation it was found that species of *Glomus* sp. and *Gigaspora* sp. dominated the AM population in all the soil sample (Fig. 2).

Histopathological study revealed the presence of vesicles and arbuscules in the root segments determining the fact that the rice roots has been infected by AMF spores (Fig. 3). Organisms of AMF have a bimodal pattern of differentiation (Morton 1990). The vegetative thallus consists of arbuscules intraradical vesicles (shared only by species in the suborder Glomineae), extra radical auxiliary cells (shared only by species in the suborder Gigasporineae), and intraradical and extra radical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham *et al.*, 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids are considered to be storage structures (Bonfante-Fasolo, 1984; Brundrett, 1991).

Under the natural condition the establishment of the brown spot disease was observed after four month growth of the rice plants grown on experimental plots (Fig.4) and Disease index (PDI%) was calculated. DI of rice cultivar Brimful was found to be the maximum with 62.28 and that of UBKV-1 to be minimum with 41.62 PDI% (Table 4).

Conclusion

The traits recorded during germplasm collection are listed on the basis of feedback from farmers and present data gives preliminary observations and require further validation after characterization /evaluation. Characterization of landraces could help breeders to utilize appropriate characters in rice improvement programme. The present investigation provides the base material for the rice breeders for exploitation of landraces possessing one or more desirable characters. The overall results of the present study have shown some of the important facts of the indigenous AM Mycorrhizal fungi present in experimental soils capable of infecting rice roots. Among the different types of AM fungi collected and observed *Glomus* sp. was found to be widely distributed in rhizosphere of rice plants in experimental plots. The present results also suggested that the rice plant may be considered as an initial stock plant which may be

used for inoculum production in departmental climatic condition. In future, the most modern and advanced technology should be considered for large-scale inoculum production of AM fungus under field condition.

Acknowledgement

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Influence of culture media and environmental factors on mycelial growth and sporulation of *Alternaria alternata* (Fr.) Keissler causing leaf blight disease of niger (*Guizotia abyssinica* (L.f.) cass)

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ABSTRACT

Alternaria alternata is isolated from naturally infected niger leaf for their morphological characteristics, mycelia growth and sporulation, spore germination in different culture media and environmental conditions. RMA was best for both growth and sporulation. Excellent sporulation was observed ion PCA. PDB supported best growth among the liquid media tested. Highest mycelia dry weight was recorded at 28°C and pH 6.5. Among several carbon sources tested, Mannitol showed optimum growth and sporulation while peptone produced maximum growth among the tested organic nitrogen sources. The present study will help to maintain the fungus in the laboratory condition for preparation of inoculums for different studies related to the control measures of the pathogen.

Key words: *Alternaria alternata*, Niger, growth, sporulation

Introduction

Niger [*Guizotia abyssinica* (L.f.) cass] is an oil seed crop and its seeds contain clear, excellent, vegetable edible oil. India is one of the important niger producing countries in the world. The oil is slow drying and it contributes about 3% of India's total oil seed production (Getinet and Sharma, 1996). In India, niger oil is frequently used as a substitute for sesame oil (Weiss, 1983). Leaf blight of niger caused by *Alternaria* sp. is the most serious disease of niger (Gebre-Medhin and Mulatu, 1992; Getinet and Sharma, 1996). The pathogen attacks the leaves. In later stage leaves of whole plant are blighted and become dark brown in colour. Fungi get food and energy from the substrate where they grow. to know their food requirements it is necessary to culture the fungus in artificial media components at optimum physical para meters that lead to maximum sporulation (Kim *et al.*, 2005; Saxena *et al.*, 2001; Saha *et al.*, 2008). The present study was under taken to assess the morphological characteristics of the fungi, the effect of different culture media, incubation periods, carbon sources, nitrogen sources, temperature and pH on the mycelia growth and sporulation of fungus *A. alternanta*, a pathogen of Niger.

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Materials and Methods

Fungal culture

The fungal culture, *Alternaria alternata* (Kr.) Keissler was isolated from naturally infected leaves of niger plants from the Barobisha region of Jalpaiguri district in West Bengal. Following verification of Koch's postulates, the organism was identified in the laboratory and our identification was further confirmed by the division of plant pathology, IARI, New Delhi (Identification no. 6250.05).

Observation of morphology

The pathogen was cultured in Potato carrot agar (PCA) and Potato dextrose agar (PDA) for ten days. A bit of fungal mycelia were taken from PDA slant and was placed on a grease free slide and stained with lactophenol and cotton blue. For study of spores, brownish mass of spores produced on the surface of the PCA slant were carefully taken out, placed on a slide and stained with lactophenol and cotton blue. Length and breadth of the spores, breath of mycelia

were measured by stage micrometer under light microscope. The detailed morphology of the fungus was noted.

Influence of different culture media

Growth and sporulation of the pathogen was first studied in nine different solid media *viz.* Richard's agar medium (RMA), Corn meal agar (CMA), Potato dextrose agar (PDA), malt extract agar (MEA), Oat meal agar (OMA), Potato carrot agar (PCA), Yeast extract mannitol agar (YEMA) and Czapak Dox Agar (CDA). Sterile petriplates (70 mm diameter) containing 10 ml of the different sterile medium were inoculated with 4 mm mycelial block. The mycelial blocks were taken from advancing zones of a PDA plate of *A. alternata* culture. The petriplates were incubated at 28±1°C for 10 days. Radial growth of mycelia was measured at 2 days intervals until 10 days. To assess the mycelia growth in liquid medium on mycelial agar block (4 mm) was transferred to a conical flask of 250 ml, containing 50 ml of liquid medium. Then Potato dextrose broth (PDB), Potato carrot broth (PCB) and Richard's medium (RM) were incubated at 28 ± 1°C. The fungal mycelia were strained through double layered cheese cloth after 5, 10, 15, 20 and 25 days of incubation and then blotted by a blotting paper and dried in hot air oven at 60°C for 24 hours. Finally, mycelial mats were cooled and dry weight was noted.

Assessment of spore germination

For assessing the spore germination the fungus was initially cultured in PCA medium for 15 days at 28 ± 1°C. Distilled water was added aseptically in the fungal culture tube, shaken and stained through a muslin cloth. The filtrate was used as spore suspension. Concentration of the spores was measured by haemocytometer count and spore concentration was adjusted by adding sterile distilled water. Thirty microliter spore suspension (1×10^{6} ml) was placed on slides and allowed to incubate for 2, 4, 6, 8, 10 and 12 hours in a humid chamber at 28 ± 1°C. The slides were stained with cotton blue-lacto phenol and observed under light microscope. Finally the percent spore germination [(no. of germination spores/ no. of spores counted) ×100)], average germ tube length in each case were calculated.

Influence of different pH

Potato dextrose broth (PDB) was adjusted to pH 5, 5.5, 6, 6.5, 7 and 8 respectively by adding 1(N) NaOH or 1(N) HCL drop-wise into the medium before sterilization. After adjusting the pH in PDB the media (50 ml in 250 ml Erlenmeyer flask) was sterilized. Media of different pH were inoculated separately by 4 mm mycelial discs of *A. alternata,* cut from advancing zone of petriplate and incubated at 28 ± 1°C. Mycelial dry weights were noted after 5,10,15,20 and 25 days of incubation.

Influence of different temperature

To assess the growth of *A. alternata* at different temperatures, the fungus was inoculated in sterile PDB media (50 ml in 250ml Erlenmeyer flask) and was inoculated at different temperatures *viz.* 10°C, 15°C, 20°C, 25°C, 28°C, 30°C, 35°C, and 40°C. After 5, 10, 15, 20 and 25 days of incubation, mycelia were harvested, strained through muslin cloth, blotted and finally dried at 60°C. Mycelial dry weights were noted. Similarly, to study the influence of different temperature on spore germination, spore suspension of *A. alternata* was prepared. Sterile distilled water was added to attain optimum concentration (1×10^{-6} ml) of spores. Spore suspension drops (30 µl) were placed in different slides in triplicates and incubated at different temperatures (10, 15, 20, 25, 28, 30, 35 and 40°C) for 2, 4, 6, 8, 10 and 12 hours.

Effect of different carbon sources on growth and sporulation

To study different carbon sources for the optimum growth and sporulation of *A. alternata,* a basal medium (Glucose 1%; Asparagine 0.2%; $MgSO_4$, $7H_2O$ 0.05%; Zn^{++}, Mn^{++} and Fe^{+++} 2µg/ml) was used for the purpose. The different carbon sources tested were glucose, sorbitol, sucrose, fructose, and mannitol. The equivalent amount of carbon present in 1% glucose was used as standard and added separately to the basal medium. The medium (50ml) was taken in

250 ml Erlenmeyer flasks and sterilized at 15 lb. p.s.i. for 15 minutes. The media was inoculated by the pathogen using 4mm mycelial discs in PDA and incubated at 28 ± 1°C for 5, 10, 15, 20 and 25 days. In control sets, no carbon sources were used in the basal medium. After incubation for the specified time periods, the mycelia were harvested, dried at 60°C and weighed. After each incubation period before harvest of mycelia, sporulation of the fungus was also recorded.

Effect of different nitrogen sources on growth and sporulation

To assess the mycelia growth and sporulation of *A. alternata* on different nitrogen sources (both organic and inorganic), modified Asthana and Hawker's medium 'A' (Glucose 10 g; KNO_3 3.5; KH_2PO_4 1.75g; $MgSO_4$, $7H_2O$ 0.75; Agar agar 20 g and distilled water 1L) without agar was used as basal medium. The quantity of various nitrogen sources was so adjusted to give the same amount of nitrogen as furnished by 3.5 g KNO_3 in the basal medium. The quantity of various nitrogen sources was prepared and dispersed separately in 50 ml medium and was taken in 250 ml Erlenmeyer flasks. The flasks were sterilized and the flasks were inoculated by test fungi using 4 mm mycelia discs in PDA and were incubated at 28±1°C for 5, 10, 15, 20 and 25 days. In control set no nitrogen source was provided in the basal medium. After specified incubation periods, sporulations were checked and were recorded. Harvested mycelia were dried at 60°C and weighed.

Statistical Analysis

Statistical analysis was done with the help of statistical package for the Social Sciences (SPSS), version 11.0, SPSS Inc. Illinois.

Result and Discussion

Microscopic study of the fungus revealed that mycelia were hyaline in colour but on maturity it becomes gray in colour. Conidia of the fungus were obclavate to beaked and brownish in colour having transverse and longitudinal septa (Fig.1a & b). Conidia were produced from simple septate conidiophores in simple or branched acropetal chains. The length and breadth of mature hyphae ranged between 3-5 µm (Fig. 1c). Similar observations regarding conidial size and shape have been reported by Maiti *et al.* (2006).

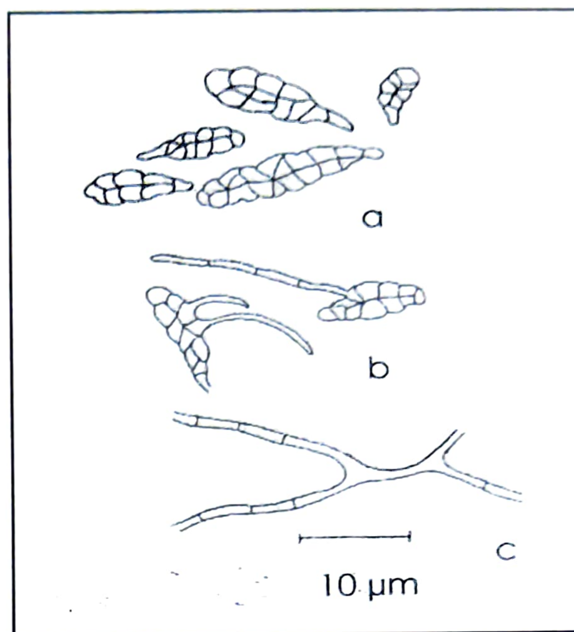

Fig. 1: (a) Spores of *A. alternata.* (b) Germinating spores with germ tubes. (c) *A. alternata* with septa and branching.

From the result presented in Table 1, it was evident that RMA (Richard's agar) was best for both growth and sporulation of *A. alternata.* After 10 days of incubation on RMA, radial growth of mycelia was 90 mm in diameter and sporulation was also good. Saha *et al* (2009) reported that best growth and sporulation of *C. gloeosporioides* was observed on PDA and RMA, which is in agreement with our findings. In PDA sporulation was comparatively less, although good growth of mycelia was evident. Our observations were also related with that Karlatti and Hiremath (1983), who found best mycelia growth of *A. zinniae* on leaf extract and potato dextrose agar media whereas *A. helianthi* showed less sporulation on potato dextrose agar (Allen *et al.* 1983; Mukewar *et al.* 1974). Excellent sporulation of *A. alternata* was observed in PCA with moderate growth of mycelia (69.33 mm in diameter). Similar results

Table 1: Mycelia growth and sporulation of *Alternaria alternata* in different solid media.

Medium for growth	Radial growth (mm)* and sporulation									
	2		4		6		8		10	
	Growth	Sp**	Growth	Sp**	Growth	Sp**	Growth	Sp**	Growth	Sp**
RMA	16.66±0.34	–	36.66±0.92	–	57.50±0.76	+	76.83±0.55	++	90.00±0.58	+++
CMA	28.16±0.57	–	14.83±0.17	–	56.16±0.74	–	70.16±0.45	++	84.16±0.45	++
PDA	13.83±0.50	–	32.40±0.95	–	50.66±0.61	–	65.83±0.44	–	82.16±0.84	+++
MEA	12.00±0.29	–	27.66±0.88	–	48.16±0.93	–	64.33±0.67	++	80.33±0.60	++
OMA	11.50±0.50	–	26.33±0.67	–	42.83±0.44	–	60.00±0.85	++	76.16±0.72	++
PCA	11.83±0.44	–	26.16±0.72	–	41.66±0.73	+	55.50±0.36	++	69.33±0.12	++++
YEMA	12.33±0.34	–	26.33±0.67	–	40.33±0.57	+	52.00±0.87	++	69.83±1.83	+++
CDA	10.33±0.17	–	15.33±0.67	–	32.16±1.09	–	31.00±1.00	+	83.33±0.88	++

*Mean of three replications, **Sp=Sporulation, – =Nil, +=poor, ++=fair, +++=good, ++++=excellent. Data after ± represent standard error values. Incubation temperature=28±1°C, CD= critical difference. PDA= Potatto dextrose agar, OMA= Oat meal agar, CDA= Czapeak Dox Agar, RMA= Richard's agar medium, YEMA= Yeast extract manitol agar, MEA= Malt extract agar, PCA= Potato carrot agar, CMA= Corn meal agar

were obtained by Prasad *et al.* (2008), who reported that growth and sporulation of *A. helianthi,* a pathogen causes leaf blight disease in sunflower, was maximum in sunflower leaf extract medium followed by carrot agar medium.

Maximum growth of *A. alternata* was recorded at 480 mg after 20 days of inoculation in PDB medium (Fig. 2). In PCB, mycelia dry weight was found 240 mg after 20 days of incubation but in RM mycelia growth was poor. After 20 days, mycelia dry weight declined due to autolysis and depletion of media.

It was found that *A. alternata* was able to grow within a wide range of pH, from 5.0 to 8.0 (Table 2). The fungus however, failed to grow in

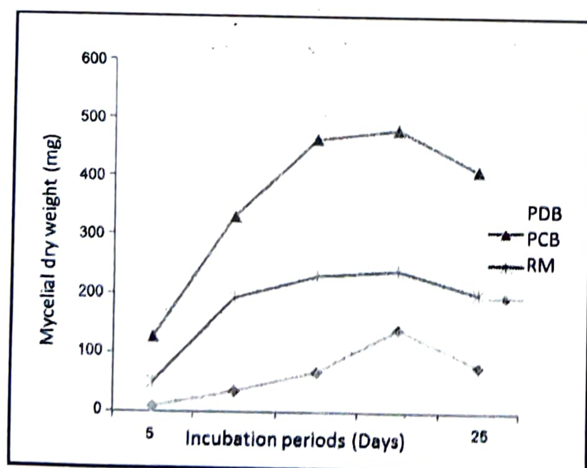

Fig. 2: Growth of *Alternaria alternata* after different incubation periods in three different liquid media. PDB= Potato dextrose broth; PCB= Potato carrot broth; RM= Richard's medium.

alkaline environment, beyond pH 8.0. Highest growth (473.4 mg) was recorded at pH 6.5. Poor growth was observed at pH 5.0 and at pH 8.0. The result indicated that slightly acidic pH to neutral pH was optimum for the growth of *A. alternata.* In a similar study, Thompson-Eagle (1989) also reported similar results where optimum pH for growth and sporulation of *A. zinnia* was found in the range of pH 6.0-6.5.

Results presented in figure (Table 3) indicate that *A. alternata* was capable of growing at temperatures that range between 10-40^0 C. Best growth was recorded at 28°C while no growth was observed at temperatures 40°C and above. These results were in agreement to those reported by Saha *et al.* (2008) who observed that optimum growth of *L. theobromae* at 28°C and no growth were noted at above 40°C. They were also in agreement with Alam *et al* (2001). Eng *et al* (2003) also reported similar observations when he studied the effect of temperature on growth characteristics of *Botryodiplodia theobromae.* He stated that the growth density and radial velocity was affected at temperature above 40°C.

During the present study, germination of spores began after 2 hours of incubation and all the spores were germinated within 12 hours (Fig. 3). Percent of germination of spores and germ tube length were recorded as 100% and 340.1 μm respectively after 12 hours of incubation. Similar results were observed by Saha *et al.* (2009), who studied the conidial

Table 2: Effect of different pH on growth of *Alternaria alternata*

pH	Mycelia dry weight (mg)				
	5 days	10 days	15 days	20 days	25 days
5.0	52.6±4.32	109.3±4.08	252.6±3.06	306.4±4.94	287.1±3.52
5.5	64.5±2.06	165.8±2.83	286.3±2.00	335.6±2.40	298.4±3.66
6.0	111.0±3.55	225.3±2.64	361.1±4.50	466.3±2.04	415.7±2.86
6.5	125.7±2.80	308.6±3.43	445.5±2.74	473.4±3.22	422.6±2.00
7.0	103.2±4.09	195.7±4.72	296.8±1.90	413.3±4.56	392.2±2.49
8.0	24.4±4.08	55.2±2.40	92.4±1.01	102.1±3.13	73.3±2.54
CD (5%)	7.12	8.53	7.63	9.50	2.60

*Mean of 3 replications: Data after ± represent standard error values. Dry weight of inoculating mycelia block was 10mg

Table 3: Effect of different temperature on mycelia growth of *Alternaria alternata*

Temperature (°C)	Mycelial dry weight (mg)*				
	5 days	10 days	15 days	20 days	25 days
10	11.2±3.24	15.1±3.33	24.8±2.36	29.5±1.53	19.7±3.47
15	16.6±2.77	22.1±3.86	32.4±3.95	38.3±1.27	29.2±4.87
20	49.0±4.60	98.7±1.17	152.3±4.41	208.4±3.84	191.3±2.27
25	89.8±4.03	182.3±3.19	241.7±3.92	369.5±4.46	345.2±4.38
28	120.3±2.51	325.1±2.36	462.7±3.79	483.4±3.88	419.5±1.40
30	70.6±3.48	151.0±4.35	189.4±2.04	224.2±3.80	213.0±4.97
35	20.2±3.56	44.7±2.55	82.4±3.39	165.6±1.54	110.5±3.48
40	15.3±3.55	34.9±2.77	55.8±3.65	64.2±2.95	51.0±3.14
CD (5%)	1.74	4.11	3.97	4.64	4.26

*Mean of 3 replications: Data after ± represent standard error values. Dry weight of inoculating mycelia block was 10mg

Table 4: Effect of different carbon sources on the growth and sporulation of *A. alternata*

Carbon source	Incubation period (Days)									
	5D		10D		15D		20D		25D	
	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**
Glucose	50.00±0.89	–	100.00±1.17	–	110.00±0.80	+	125.00±0.90	++	100.00±1.29	+++
Sucrose	110.60±0.99	–	200.00±1.15	+	340.40±1.14	++	410.60±0.66	+++	385.00±0.68	++++
Fructose	100.00±1.04	–	190.10±0.95	–	310.2±1.25	+	420.40±0.76	++	390.00±1.46	+++
Sorbitol	80.00±0.81	–	170.60±0.59	–	280.3±0.85	+	300.40±0.83	++	310.00±0.87	++
Mannitol	110.50±0.45	–	240.80±0.85	+	380.90±0.87	++	500.00±1.29	++++	495.80±0.31	+++
Control***	10.30±1.08	–	12.60±0.95	–	13.90±0.03	–	14.10±0.80	–	13.11±0.49	–
CD (5%)	2.03		2.01		12.55		1.66		2.58	

*Mean of three replications. Sp=Sporulation, –=Nil, +=poor, ++=fair, +++=good, ++++=excellent. ***Control Basal medium without any carbon source. Mwt. (mg)= Mycelial dry weight in mg; Data after ± represent standard error values.

germination and appresoria formation in the brinjal anthracnose causing fungus *C. gloeosporoides.* They reported germination and appresoria formation within a period of 8 hours. Spore germination of *Bipolaris carbonum* was also observed to begin within 2-4 hours *in vitro* by Saha and Chakraborty (1990). Effect of temperature on spore germination was studied and results are presented in Fig. 4. Result revealed that spore germination was optimum at

28°C (100% after 12 hours) whereas 2.45% spore germination occurred at 10°C after 12 hours but no germination occurred at 40°C or above.

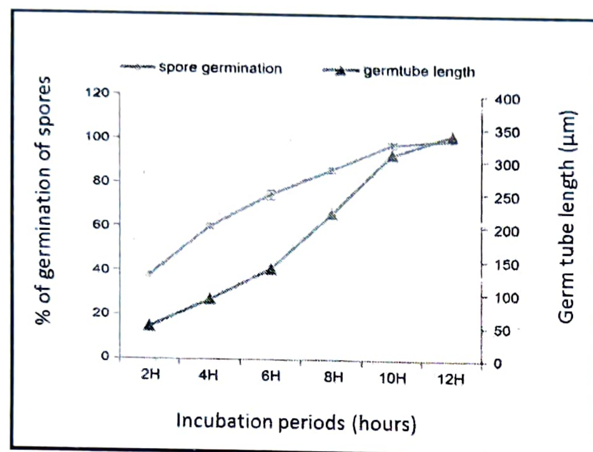

Fig. 3: Effect of different incubation periods on spore germination and germ tube elongation.

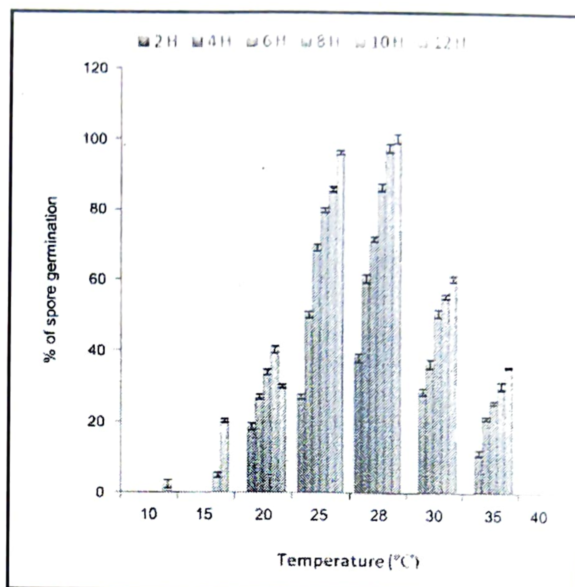

Fig. 4: Effect of different temperatures on spore germination of *A. alternate.*

Nutritional requirements of the pathogen were studied and it was concluded that mannitol was the best carbon source for optimum growth and sporulation of *A. alternata* (Table 4). Next to mannitol, good growth was observed in fructose and sucrose respectively. When nitrogen sources were tested, peptone produced best growth and sporulations, while potassium nitrate showed best growth among the tested inorganic nitrogen sources (Table. 5). Several workers (Devdath *et al.,* 1977; Jadhav *et al.,* 2002; Jash *et al.,* 2003) studied the influence of various carbon and nitrogen sources on fungal metabolism. Jadhav *et al.,* (2003) observed that highest mycelia growth and sporulation was recorded when mannitol was used as carbon source and peptone was used as nitrogen source. Wu and Wu (2003) observed that *Alternaria protenta,* a pathogen of sunflower showed abundance of sporulation on glucose

Table 5: Effect of different nitrogen source on the growth and sporulation of *A. alternata*

Carbon source	Incubation period (Days)									
	5D		10D		15D		20D		25D	
	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**	Mwt. (mg)	Sp**
					Inorganic					
Potassium nitrate	150.00±1.00	-	220.50±0.66	+	265.2±0.70	+++	286.00±0.98	+++	250.00±1.32	+++
Sodium nitrate	130.60±0.74	-	186.20±0.76	+	210.40±0.90	++	275.00±0.92	++	225.00±0.76	++
Ammonium sulphate	120.80±0.53	-	163.40±0.83	+	198.00±0.81	++	245.10±0.72	++	215.00±0.72	++
					Organic					
Peptone	230.10±1.27	-	298.60±0.61	+	340.00±1.53	++	360.00±1.25	++	310.00±1.15	++
Yeast extract	210.40±1.00	-	250.00±1.26	+	290.30±1.12	++	340.00±1.44	+++	300.00±1.15	+++
Beef extract	115.00±0.81		165.00±1.63	-	200.60±0.70	++	235.40±0.72	++	215.00±0.72	++
Control***	5.90±0.32	-	910±0.92	-	10.60±0.31	-	11.50±0.55	-	14.80±0.67	-
CD (5%)	1.58		2.05		1.32				0.77	

Mwt. (mg)= Mycelial dry weight in mg; *Mean of three replicates. Data after ± represent standard error values. Sp=Sporulation, −=Nil, +=poor, ++=fair, +++=good, ++++=excellent. ***Control Basal medium without any carbon source.

peptone agar but not on dextrose nitrate agar.

The result of the present study is crucial for further studies of the fungus as a pathogen of niger and supplemented media as suggested here may be utilized for inoculum production.

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In vitro seed germination of an Endangered Terrestrial Orchid Species *Geodorum densiflorum* (Lam.) Schltr.

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Abstract

Orchid seed is rather difficult to germination rather than other angiospermic seeds, because of lack endosperm, radical and leaf rudiments. Seed germination and protocorm like body (PBL) formation of *Geodorum densiflorum* (Lam.) Schltr. was performed. This study was conducted to determine the effects of coconut milk (CM), and BAP for optimal media culture. Seeds from sterilized capsules were cultured on two media (Knudson C orchid medium, KnC, and MS medium) containing 0 and 25% (v/v) CM and 0 and 5 mgl^{-1} BAP with 0.8% (w/v) agar as solidifying material. The cultures were maintained at 25±2°C with 12-hour illumination of 60 µmoles m^{-2} sec^{-1} light intensity provided by cool white florescent tubes. The highest germination percentage was observed in 15% (v/v), CM and 3 mg l^{-1} BAP both in MS and KnC media. Seeds germinated and formed light green globular structures on the medium after three weeks of culture. These globular structures produced Protocorm-like body (PLB) and proliferated and developed into irregular-shaped rhizomes with white hairy structures. Highest *in vitro* seed germination was found in KnC medium supplemented with 15% CM and 3 mgl^{-1} BAP about 95.31 % whereas in MS medium maximum germination reached at 79% with 15% CM and 3 mg l^{-1} BAP. Overall, this study showed that *Geodorum* seeds cultured on KnC medium containing CM and BAP can be used for clonal propagation.

Introduction

Orchids belonging to the family Orchidaceae are one of the largest and most evolved flowering plants. Orchidaceae includes about 800 genera and between 25,000 to 30,000 species distributed all over the world (Chowdhery, 2001). Orchids produce one of the most beautiful and enchanting flowers that have fascinated people of all ages. Besides being considered an ornamental treasure in the commercial market, orchids have important medicinal properties used in the preparation of herbal medicines in different parts of the world (Arditti, 1992). According to World Health Organization, 80% of people depend mainly on traditional remedies such as herbs for medicine (Kala, 2005), resulting in increasing demand for medicinal plants. Orchids are experiencing a steady decline in tropical countries due to destruction of natural forest areas. It is essential to take measures for the conservation and propagation of these endangered orchid species (Hossain, 2015; Hossain *et al.*, 2013; Hossain and Dey, 2013). *Geodorum densiflorum* (Lam.) Schltr. is an endangered terrestrial orchid appearing above the ground only during the rainy season. The introduction of an asymbiotic seed germination method by Knudson (1946) and shoot tip culture by Morel (1960) has helped in developing methods for orchid propagation. *In vitro* culture of seeds of *Geodorum densiflorum* (Lam.) Schltr. resulted in development of protocorms. *Geodorum densiflorum* is one of the floriculturally and medicinally important ground orchids. Rhizomes of *G. densiflorum* are used as medicine for the treatment of various diseases (Rao 1979). Because of damage of its natural habitats by continuous destruction of forest for land reclamation and indiscriminate collection by orchid lovers, this species has now become endangered. But the demand of such orchids is increasing day by day in local and foreign markets. As orchid seeds do not possess endosperm, their natural germination is limited and need a symbiotic association with specific mycorrhizal fungus. The discovery of *in vitro* seed germination and micropropagation contribute immensely to alleviate their scarcity. Sheelavantmath *et al.* (2000) reported a protocol for rhizome based propagation of *G. densiflorum*. Growth rate of the tissues can be increased by the addition of organic supplements and plant extracts. Many different

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organic additives like coconut water, banana pulp, peptone, tomato juice, slap honey, date palm syrup, corn extract, papaya extract and beef extract influenced medium culture and provide undefined mixture of organic nutrients and growth factors (Gnasekaran *et al.*, 2012 and Nambiar *et al.*, 2012; Shekarriz *et al.*, 2014). Coconut milk is the extract of white, solid endosperm of matured coconut after grinding and squeezing. This compound is often used when no other composition of known defined components produces the desire growth or development. Coconut milk (CM) is the colorless liquid endosperm of green coconuts (*Cocos nucifera*) which its liquid endosperm contains a number of amino acids, organic acids, nucleic acids, several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals and other unidentified substances, none of which alone is totally responsible for growth promoting qualities (Lu 2013).

The present investigation was undertaken with a view to developing an efficient *in vitro* cultural technique of germination, possibility of inducing PLBs from the seeds and micropropagation of *Geodorum densiflorum*, an endangered orchid species of North Bengal to help in *ex situ* conservation.

Materials and Methods

Mature capsules of *Geodorum densiflorum* were surface sterilized by submerging them in a 0.1% (w/v/) $HgCl_2$ (mercuric chloride) solution for 8 mins with occasional agitation followed three washes in sterilized distilled water. Then dip in absolute ethanol for 20-25 sec. The sterilized capsules were then washed thrice with sterile distilled water. The capsules were then cut with a sterile surgical blade and the seeds were inoculated on to the surface of the Knudson C medium (Knudson, 1946) and MS medium (Murashige and Skoog, 1962) with growth regulators and adjuvants. All works were performed in a laminar airflow cabinet. The pH of the media was adjusted to 5.8 with 0.1N NaOH or 0.1N HCl prior to autoclaving and the medium was solidified with 0.8% agar. Media was autoclaved at 121°C for 15 mins at 15 psi. The cultures were maintained at 25±2°C with a cycle of 12/12 hour continuous light (illumination of 60 µmole $m^{-2}s^{-1}$) and dark conditions provided by cool white florescent tubes (Philips India). After germination of seeds, protocorms were subcultured at 25-day interval. Two different types of media were used in the present investigation KnC and MS media. Media were solidified with 0.8% (w/v) agar and fortified with different concentrations and combinations of PGRs (0-5 mgl^{-1} BAP and 0-25% coconut milk) were used for the purpose. Three replicates per treatment, arranged in a completely randomized design (CRD) were maintained. Survival percentage, germination percentage and protocorm like body formation were recorded.

Results and Discussion

Seed Germination and PLB formation

The seeds germinated on all the nutrient media used but germination percentages varied depending on the media composition. Maximum seed germination (95.31%) was recorded in KnC medium when fortified with 3.0 mgL^{-1} BAP + CM 15%. Species-specific media for seed germination have been reported in orchids (Arditti and Ernst, 1984). The effect of CM and BAP and media culture on seed germination

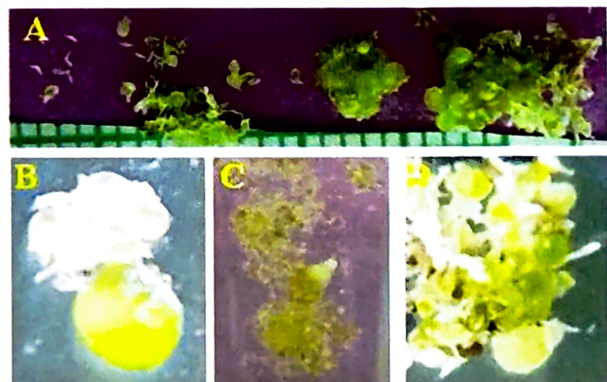

Fig. 1: In Vitro seed germination of one endangered orchid species *Geodorum densiflorum*. A. Seed swelling and embryo rupture from the seed coat, B. Globular structure before PLB formation, C. Protocorm like body formed (PLBs) and D. Secondary PLBs are formed

percentage is shown in Table 1. CM and BAP and media culture were significantly influenced germination percentage in *Geodorum*. Germination in presence of CM was better than

control. Germination increased when added coconut milk in both the media KnC and MS (Table 1). The specificity was reported even within species of the same genus, for example, Mitra *et al.* (1976) medium for *Cymbidium macrorhizon* (Vij and Pathak. 1988; Jamir *et al.*, 2002) medium for *C. iridioides* and Knudson C for *C. elegans* (Sharma and Tandon 1990). After 2 months, the embryos swelled and broke out of the testa, and then formed protocorm like-bodies (PLBs) (Figure 1) by 4 months. The protocorm enlarged, and produced rhizomes with multiple buds (Figure 1). The four month old PLBs were sub-cultured in the same medium for secondary PLBs formation for micropropagation purpose.

The protocorms proliferated in germination media but these did not develop seedlings in the culture media. Germination of orchid seeds followed a peculiar metamorphogenetic pathway (Figure 1); that is, undifferentiated embryos swelled up by absorbing water and nutrients from the media and developed a compact mass of parenchymatous cells called spherule which gradually develop protocorm, an intermediate structure between seed and seedling (Leroux *et al.*, 1997). At the initial stage of protocorm development (Figure 1), an appendicle, looking like a closed ridge, appeared at the upper part of the protocorms which leads to shoot formation while basal part escorts root development (Hossain and Dey, 2013). The protocorms became elongated and formed rhizome-like bodies (RLBs) with numerous hairs and some growth appendages on the body surface and a growing tip indicating the development of leafy shoots and the root initials, respectively. Formation of RLBs in *in vitro* protocorms has also been reported in a terrestrial orchid, *Geodorum densiflorum* (Bhadra and Hossain, 2003). Kanjilal and Datta (2000) reported that peptone (2 g l^{-1}) was effective in promoting the survival percentage of explants a terrestrial orchid *Geodorum densiflorum* (Lam) Schltr. but had no effect on PLB production. BAP is known to enhance germination frequency in *Cypripedium* spp., *Eulophia dabia*, and *Pachystoma senile* and stimulated protocorm multiplication as well as shoot formation in *Cymbidium pendulum* and *Cattleya aurantiaca*. This is a simple and efficient procedure for seed germination and PLB formation of *Geodorum densiflorum* (Lam.) Schltr. could be used for large-scale propagation and *ex situ* conservation of this endangered orchid species. PBL formation in different culture media significantly affected PBL formation in *Geodorum* orchid but CM had shown significant effect on PBL formation. The highest PBL number was showed with 15 % CM with 3 mgl^{-1} BAP in compare to control (0 mgl^{-1}). Between media culture, KnC medium showed the highest PBL formation (Table 1).

Table 1. Seed germination of one orchid species *Geodorum densiflorum.*

Medium	PGRs (mgL^{-1}) BAP	Additives (CM %)	Time (days)		Seed germination
			Spherule	Protocorms	(%) (mean ± SE)
MS	0.0	0	30-40	40–45	53.00 ± 1.34
	1.0	5	30–40	40–45	61.00 ± 2.30
	2.0	10	25–32	35–40	68 .00 ± 1.37
	3.0	15	20–25	30–35	79.00 ± 0.92
	4.0	20	25–35	40–45	65.00 ± 2.01
	5.0	25	30–40	40–45	55.00 ± 1.44
KnC	0.0	0	20–25	40–45	65.57 ± 1.22
	1.0	5	25–30	45–50	71.08 ± 1.58
	2.0	10	20–25	35–40	84.00 ± 1.09
	3.0	15	20–25	30–35	95.31 ± 0.08
	4.0	20	20–25	35-40	76.77 ± 1.99
	5.0	25	20–25	40-45	70.34 ± 2.03

Mean values of three replicates ± Sd.

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A comparative study of silkworm (*Bombyx mori* L.) rearing under different sources of peptides isolated from Dudhiya and S1 mulberry leaves

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Abstract

Silkworm is a domestic monophagous insect, produces only natural animal fibre. Growth of silkworm larvae depends on the nutritional components of mulberry leaves. In present study one attempt was made to investigate the role of low molecular weight (0.5-3 kDa) mulberry peptides on silkworm rearing. For peptide extraction, two different types of mulberry leaves were chosen, one from S1 which was preferred by larvae for feeding, another is a germplasm named Dudhia, refused by larvae. Peptides isolated from young S1 leaves showed higher larval growth followed by peptides isolated from mature and senescence leaves. High ERR% along with enhanced weight of single cocoon and single shell was observed in silkworm fed with S1 peptide treated mulberry leaves as compared with the same by Dudhia peptides. Elevated antioxidant activities were exhibited by S1 peptides than Dudhia at all maturity stages (young, mature and senescence). Significant correlation was obtained between antioxidant activities of S1 peptides and economical attributes of silkworm rearing such as ERR %, weight of single cocoon, weight of single shell etc. From our observation it might be stated that the farmers would have been benefited if they could use mulberry leaves treated with antioxidant enriched peptides as a food for silkworm rearing.

Keywords: Oligopeptides, *Bombyx mori,* HPLC, antioxidant, Mulberry leaf.

Introduction

The *Bombyx mori* L. is an important sericigenous insect due to their golden fibre and it plays an important component of sericulture industry which contributes to the economic development of India and Bangladesh. The nutritional quality and quantity of mulberry leaves have a direct consequence on silkworm growth and development and subsequent cocoon production (Seidavi *et al.* 2005). Recently scientists are trying to improve silkworm rearing by feeding them different mulberry leaf supplementary products. The effects of different types of dietary protein on silkworm growth were determined by using semi-synthetic diets. Several reports stated that protein acts as an essential ingredient in silkworm diet (Horie and Watanabe 1983; El-Sayed and Nagda 1999). Smaller proteins less than 10 kDa have also been considered as peptides, therefore it can be predicted that these peptides might also have significant impact on the growth and development of silkworm. In present study, a scientific attempt was made to find out the effect of peptide(s) at low molecular weight ranges (0.5-3 kDa) isolated from mulberry leaves. Dudhia is a germplasm of mulberry and S1 is a cultivar used for peptides extraction at different maturation stages. As silkworm larvae have feeding preference on leaves of S1 cultivars than Dudhia germplasm (primitive), this study was undertaken by comparing the rearing efficiency and antioxidant activity of oligopeptides isolated from the two above mentioned sources of mulberry leaves.

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Material and methods

Plant culture

Leaves of S1 cultivars of mulberry and Dudhia were collected from Sericulture Farm of Malda, West Bengal, India. Leaves were selected at different maturity stages namely young, mature and senescence leaves at same season and same time. Young, mature and senescence leaves were selected on the basis of the biochemical attributes (chlorophyll and protein content) and the morphological parameters (length and breadth) of the leaves.

Isolation and purification of low molecular weight peptide(s)

Mulberry leaves from Dudhia germplasm and S1 cultivar amounting 1 kg each at three different maturation stages were surface sterilized. For peptide isolation the leaves were separately crushed with liquid nitrogen by a grinder and extracted with a measured amount chilled distilled water by blender at 4^0 C. The extract was cold centrifuged at 10,000 rpm for 30 minutes using protease inhibitor PMSF. The supernatant was subjected to ether wash at acidic pH to remove endogenous hormonal impurities, fats, lipids and oil as impurities. It was then passed through separate cation exchanger resin (Dowex-50; 900 meq. in glass column 60 × 2.9 cm) to get anionic hormone free solution, like indole 3-acetic acid (IAA), abscisic acid (ABA) and gibberellic acid (GA_3). The sample obtained after cation resin was dissolved in water with little acidic pH and again passed through anion resin (700 meq-Sigma Chemical Co. USA filled in glass column 60 cm × 2.9 cm, 1.6 meq / ml) for trapping amphoteric molecules like proteins, peptides and amino acids. Then concentrated aqueous acidic column eluents were washed 4 times with equal volume of peroxide free ether to remove traces of IAA, ABA, and GA_3. After discarding anionic hormones, the extracts were filtered through Millipore Ultrafiltration device with 10 kDa (YM 10), 3 kDa (YM3) and 0.5 kDa (YC 05) cut off ultrafiltration membranes (Amicon made) under 1.5 kg/cm^2 N_2 gas pressure. The samples were repetitively filtered and lyophilized. The obtained peptide extract was dissolved in 50 mL distilled water and stored in freeze at -20° C for further analysis.

HPLC analysis

The semi purified concentrated peptide(s) from different maturity status of leaves were passed through C_{18} HPLC Waters™ 486 reverse phase column with 10% methanol as running solvent fitted with 515 HPLC pump, runtime 60 minutes, absorbance at 250 nm, column length 3.9 × 150 mm, injection volume 20µl, flow rate 0.5-1.0 ml / minutes with pump pressure of 4000 psi. The peptide(s) appeared at different retention time were repeatedly loaded and purified, concentrated *in vacuo* and stored in deep freeze under -20^0 C. Each peak was isolated with their retention time and re-injected into the column to check its repetitive occurrence.

Sequencing of bioactive peptide separated through HPLC was performed through Shimadzu PPSQ-31A automated protein sequencer with 15 cycles operation, reactor temperature 60^0C, column temperature 37^0 C with mobile phase by 10% methanol. HPLC characterization of PTHs made use of a steel-walled C_{18} analytical column. After each cycle of Edman degradation, the PTH-derivatives were identified through Shimadzu UV-Vis SPD-20A Detector with detecting wavelength at 289 nm. System integrator calibrated the maximum probable sequence of amino acids.

Feeding trail

Present experiment was conducted in the laboratory under optimum temperature (27^0-29^0 C) and humidity (70 ± 5%). For the diseases free laying (DFL) of silkworm rearing, 5th instar F1 hybrid (Nistari × bivoltine) of silkworm larvae were selected and reared according to Jha *et al.* (2014). As a control, fifty larvae were reared by feeding with S1635 mulberry leaves (mulberry stem shocked under distilled water for 4 hrs). Side by side similar experiment was conducted with peptides treated S1 mulberry leaves separately. Peptide(s) isolated from young (P_y), mature (P_m), and senescence (P_s) leaves in both range 0.5-3 kDa and 3-10 kDa was diluted 20 times by distilled water. Leaves were soaked in peptide(s) for 30 minutes before feeding them to the larvae and air-dried and given to silkworm. Six separate groups, with 10 larvae were kept and fed by different peptide(s) treated leaves in separate plastic tray. Each experimental set was highly maintained contamination free. Each four hours duration larvae were fed by elicited leaves (4 times every day) until cocoons formation would be started. The larval weight was recorded by weighing them each day and growth rate pattern of the larvae was calculated. After cocoon formation, the cocoon weight of each set was recorded. When moths were released out from cocoon, cocoon shell weight was also measured. Growth rate, effective rearing rate (ERR), shell ratio, weight of single cocoon and single shell

was calculated by formulae used in Jha *et al.* (2015).

$$\text{Shell ratio (\%)} = \frac{\text{Single shell weight (gm)}}{\text{Single cocoon weight (gm)}} \times 100$$

$$\text{ERR \%} = \frac{\text{Total no. of cocoons harvested}}{\text{Total no. of larvae brushed}} \times 100$$

$$\text{Weight of single cocoon} = \frac{\text{Weight of 5 male cocoons} + \text{Weight of 5 female cocoons (gm)}}{\text{No.of cocoons taken (10)}} \times 100$$

$$\text{Single shell weight} = \frac{\text{Total shell weight of 5 male cocoons} + \text{5 female cocoon shells (gm)}}{\text{Total no of cocoons taken (10)}} \times 100$$

Determination of antioxidant activity of isolated peptide(s)

$ABTS^+$ scavenging activity

The spectrophotometric analysis of $ABTS^+$ radical cation(s) scavenging activity was determined according to Re *et al.* (1999) method with some modifications. The $ABTS^+$ was obtained by reacting 7 mM $ABTS^+$ radical cation(s) in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 12-16 hrs. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.750 ± 0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 2 mL of $ABTS^+$ solution was added to 1 mL of the aqueous extract. After 30 min, absorbance value was recorded at 734 nm, relative to a blank absorbance. The percentage inhibition of the samples was calculated as:

Inhibition % $= (1 - A/A_0) \times 100$

Where A_0 is the absorbance at 734 nm of the control, *A* is the absorbance at 734 nm of the sample mixture.

DPPH Scavenging activity

Antioxidant activity of LMW peptide was examined by using capacity of free radical scavenging effect of stable DPPH free radical. The radical scavenging activity of the aqueous extracts was measured by DPPH method (Blois 1958). In this assay ascorbic acid was used as a standard compounds. The absorbance was measured at 517 nm. A reaction mixture without test sample was taken as control. The free radical scavenging activity of tested sample were expressed as percentage of inhibition and were calculated according to these equation:

% inhibition of DPPH activity$=[(A_0-A_1)/A_0]\times 100\%$

Where A_0 is the absorbance values of the blank sample i.e. control reaction and A_1 is the absorbance value of the tested sample. A curve of inhibition percent or percent scavenging rate against sample concentrations was determined from where IC_{50} (concentration of the sample required to inhibit 50 % of free radicals) of tested sample were calculated.

Reducing power

The assay was performed according to the method of Oyaizu (1986) with some modifications. To determine reducing power activity of peptide, 1% potassium ferricyanide solution was used. Fluorescent green colour was appeared and absorbance of the final solution was recorded at 700 nm.

Nitric oxide Scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci 1994). For this reaction 320 µL extract, 360 µL (5 mM) sodium nitroprusside-PBS solutions, 216 µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthyl ethylene diamine dihydrochloride) was mixed and incubated at 25°C for one hour. Finally 2 mL water was added and absorbance was taken at 546 nm.

Superoxide anion radical scavenging activity

The superoxide radical scavenging activity was measured by the method of Nishikimi *et al.* (1972) with slight modifications. The reaction mixture contained 1 mL of NBT solution, 1 mL of NADH solution and 1 mL of methanolic extract of different concentrations. After 5 min incubation, 100 µL of PMS was added to the reaction mixture. The reactant was illuminated at 25°C for

30 min and the absorbance was measured at 560 nm against methanol as control.

Result and Discussion

Comparative analysis of effects of peptides on silkworm rearing system

The larval growth and development depends on the essential nutrients in exact ratio (Kanafi *et al.* 2007). It was earlier reported that mulberry leaves supplemented with different nutrients

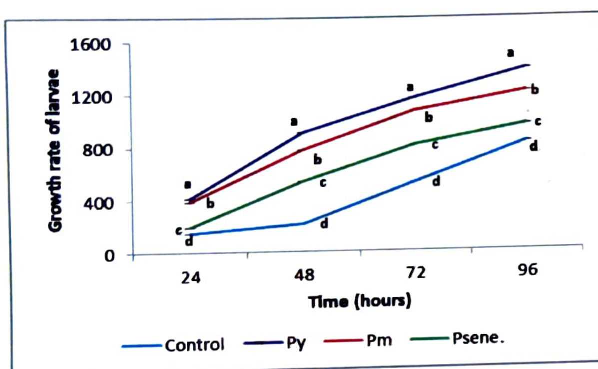


Figure 1: Growth rate of larvae in S1 leaves treated with 0.5-3 kDa peptide isolated at different maturity stages of leaves of S1 and control (only S1 leaves). Results are represented as mean ± SEM, n = 3. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

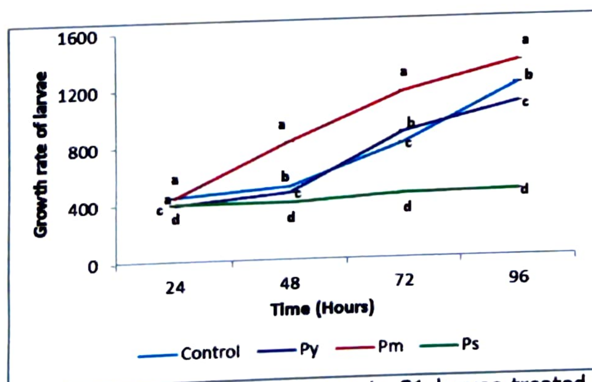


Figure 2: Growth rates of larvae in S1 leaves treated with 0.5-3 kDa peptide isolated at different maturity stages of leaves of Dudhia and control (only S1 leaves). Results are represented as mean ± SEM, n = 3. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

improved silkworm larval growth (Sarker *et al.* 1993). Also previous findings supported that peptides at low molecular weight range had an effect on silkworm larval growth and silk production (Jha *et al.* 2014, 2015). Paralytic peptides of silkworm larvae had *in vitro* effects on both in hematopoietic regulation and silkworm larval haemocyte immune reaction (Nakahara *et al.* 2003). Literature survey also reveals that oligopeptides from different plants had a role on larval growth and their metabolic activity. Our study has unveiled that low molecular weight S1 mulberry peptides influence the larval growth and other economic attributes of silkworm rearing system. Dudhia peptide showed comparatively reduced effects on larval growth than S1. On the other hand, effects of Dudhia peptides significantly improved over control. These observations clearly indicate that short protein or peptides can influence the silkworm larval growth and silk production and also it was source dependent. Highest larval growth rate was recorded at 96 hrs after 4th moulting under all peptides treatment. Similar occurrence was also reported earlier by Jha *et al.* (2014). Larval growth rate was gradually increased from 24 hrs to 96 hrs under all peptides treatment as well as in control (Figure 1). In case of S1 peptides treatment, larval growth and economic attributes of Py (peptides isolated from young leaves) was followed by Pm and Ps (peptides isolated from mature and senescence leaves respectively), and all of their bioactivities improved over control. Conversely when Dudhia peptides were considered, only Pm showed effective growth rate over control but Py and Ps had no positive effects (Figure 2). Weight of single cocoon and single shell was high under Py treatment of S1 peptides followed by Pm and Ps (Table 1). In case of Dudhia peptide treatment, highest economic attributes like cocoon weight and single cocoon shell weight was recorded under Pm treatment followed by Py and Ps. Mulberry leaves supplemented with soybean flour had effects on larval weight. Sridhar and Radha (1986) found significant larval growth and enhanced economic attributes after feeding mulberry leaves treated with amino acids. Amala Rani (2011) reported that mulberry leaves supplemented with protein had significant effect on larval growth and different economic parameters of silkworm rearing system. Our experimental data stated that low molecular weight peptides influenced the

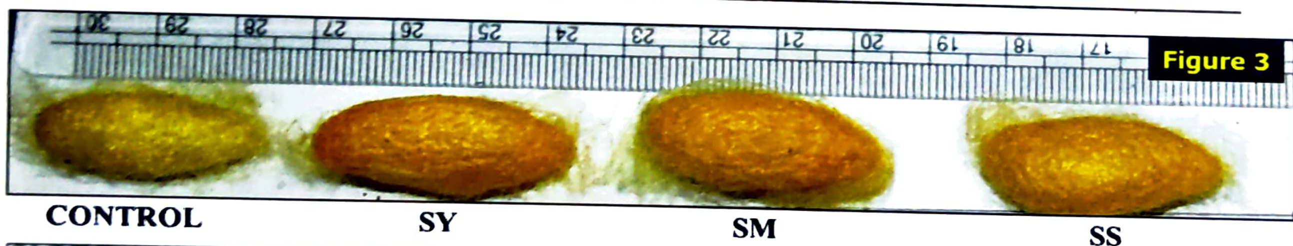
economical parameters of the silkworm rearing system. Figure 3 and 4 shows the comparative accounts of cocoons obtained after S1 and Dudhia peptide treatment.

Comparative studies of antioxidant activity of isolated peptide(s)

The results of ABTS$^{\cdot+}$ and DPPH free radical-scavenging activity of purified peptides are shown in Figure 5 and 6 respectively. Both free radical-scavenging activity of peptide samples isolated from Dudhia and S1 mulberry leaves, increased in a concentration-dependent manner. High scavenging activity was recorded in SY (peptides isolated from S1 young leaves). Low IC_{50} values mean high antioxidant activities. In case of DPPH and ABTS$^{\cdot+}$ scavenging assay, S1 showed higher scavenging activity than Dudhia at each maturity stages. Peptides isolated from young leaves exhibited higher antioxidant activity than Pm and Ps in both sources of mulberry leaves. Nitric oxide is responsible for numerous physiological processes like vasodilation, immune response, neural signal transmission etc (Wink *et al.* 1991). Our experiments revealed that Pm had higher nitric oxide scavenging activity rather than Py and Ps in both Dudhia and S1 peptides (Figure 7). Superoxide is considered as an initial free radical, formed from mitochondrial electron transport systems and creates other cell-damaging free radicals, such as hydrogen

Table 1: Effect of S1 and Dudhia peptides on various economic attributes of silkworm rearing system (where SY, SM & SS means peptides isolated from S1 young, mature and senescence leaves respectively and DY, DM & DS means peptides isolated from Dudhia young, mature and senescence leaves respectively).

Treatment	Weight of single cocoon	Weight of single Shell	Shell ratio (%)	ERR (%)
Control (S1 leaves)	0.66 ± 0.027	0.098 ± 0.019	14.85	100.00
SY	0.78 ± 0 .015	0.178 ± 0.02	22.82	100.00
SM	0.72 ± 0.018	0.115 ± 0.01	15.97	100
SS	0.74 ± 0.016	0.12 ± 0.005	15.75	90
DY	0.688 ± 0.018	0.16 ± 0.011	23.84	41.67
DM	0.71 ± 0.009	0.114 ± 0.007	16.06	83.33
DS	0.57 ± 0.006	0.113 ± 0.01	19.82	58.33



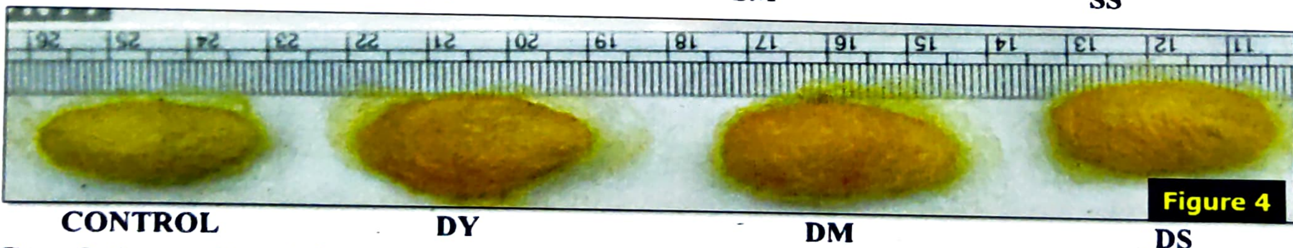


Figure 3: Cocoon obtained after S1 peptide treatment and from control (after untreated mulberry leaves). Here, SY, SM and SS stands for peptides isolated from young, mature and senescence leaves of S1 mulberry leaves respectively.
Figure 4: Cocoon obtained after Dudhia peptide treatment and from control (after untreated mulberry leaves). Here, DY, DM and DS stands for peptides isolated from young, mature and senescence leaves of Dudhia leaves respectively.

peroxide, singlet oxygen or hydroxyl radical (Bloknina *et al.* 2003). Peptides can protect the cells against toxic effect of some superoxide free radicals (Comfort *et al.* 2011).The results shown in Figure 8 clearly indicates that peptides isolated from S1 young and mature leaves have high potential superoxide scavenging activity than the Dudhia peptides.

The reducing capacity of a biological compound plays a significant indicator of its potential antioxidant activity in reducing power determining assay (Kallithraka *et al.* 2001). As shown in Figure 9, Py showed high antioxidant activity than Pm and Ps in case of both. All peptides isolated from S1 leaves exhibited higher antioxidant activity than their respective counterparts of Dudhia peptides.

IC_{50} values of S1 peptides like DPPH, $ABTS^{\cdot+}$, nitric oxide, superoxide was negatively correlated with different economical parameters of silkworm rearing system such as ERR%, WSC (weight of single cocoon), WSS (weight of single shell) etc. As the IC_{50} values of different free-radical scavenging components were negatively associated with antioxidant property, economic attributes were directly related to antioxidant activity of isolated peptides, which means that antioxidant rich peptide from mulberry leaves might elicit the growth of silkworm and facilitate metamorphosis from larval stage to pupa as well as cocoon production.

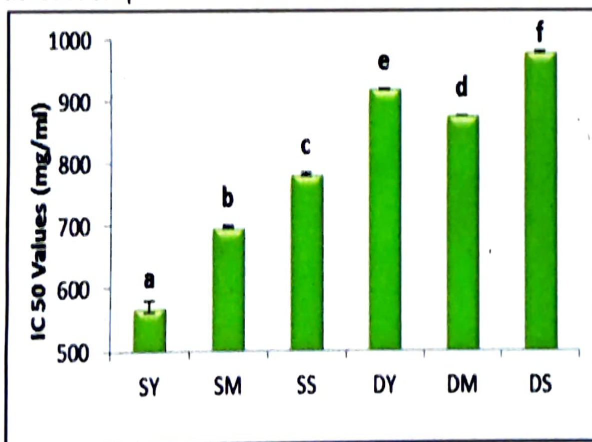


Figure 5: Comparative analysis of $ABTS^{+}$ scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-f) are significantly (P < 0.05) different from each other by Duncan's multiple range test (DMRT).

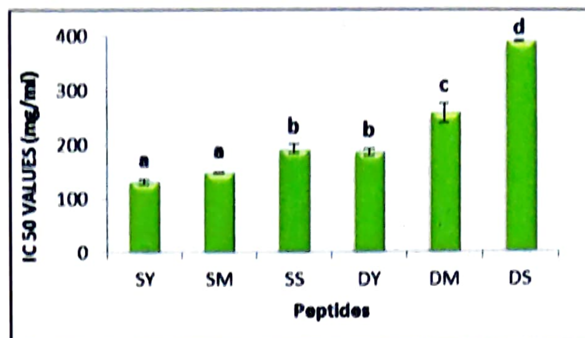


Figure 6: Comparative analysis of DPPH scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-d) are significantly (P < 0.05) different from each other by Duncan's multiple range test (DMRT).

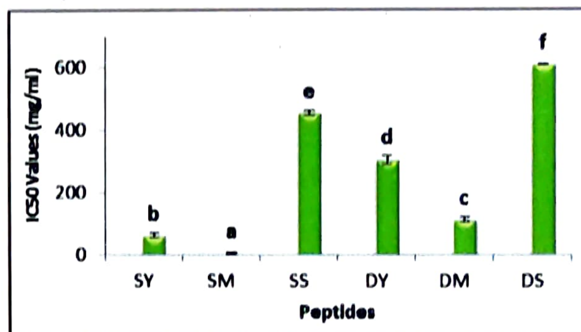


Figure 7: Comparative analysis of nitric oxide scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-f) are significantly (P < 0.05) different from each other by Duncan's multiple range test (DMRT).

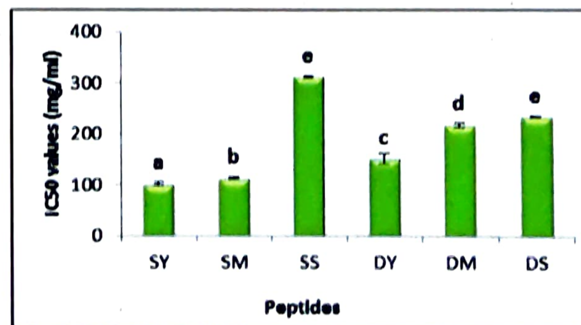


Figure 8: Comparative analysis of superoxide scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-e) are significantly (P < 0.05) different from each other by Duncan's multiple range test (DMRT).

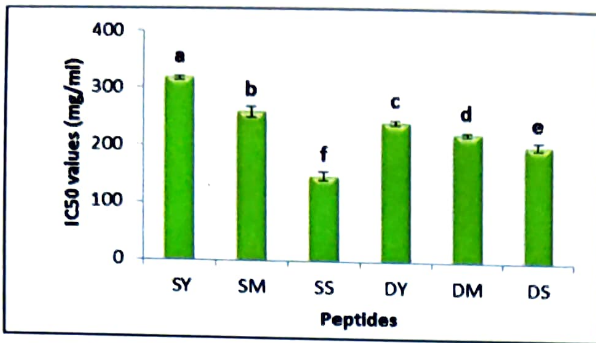


Figure 9: Comparative analysis of superoxide scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-e) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

HPLC and peptide(s) sequencing

Based on the retention time, isolated heterogeneous oligopeptides from different maturity stages of leaves exhibited different pattern in High Performance Liquid Chromatographic (HPLC) profile. Figure 10 (a and b) showed the HPLC chromatogram of Py from S1 and Dudhia respectively. As Pm and Ps of S1 showed better response in silkworm feeding and the same of Dudhia counterparts does not having remarkable effects on silkworm rearing, the comparative profile of these two peptides isolated

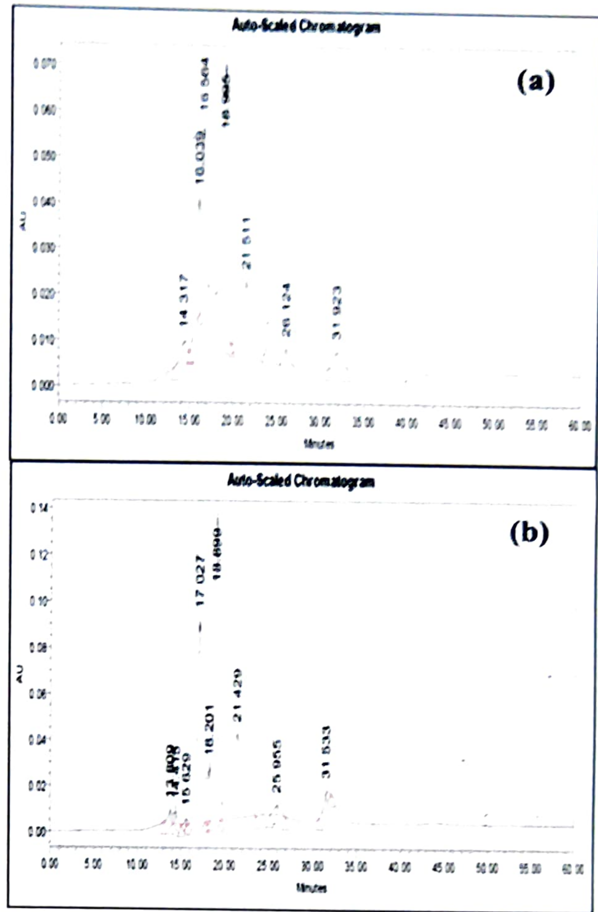
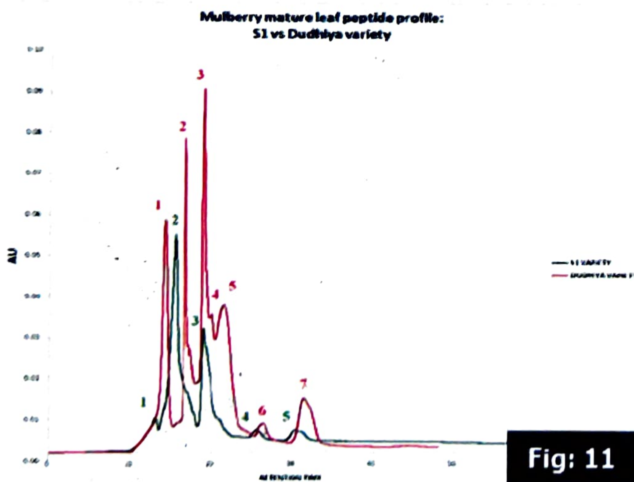


Figure 10: HPLC generated auto-scaled chromatogram of peptide(s) isolated from (a) S1 and (b) Dudhia young leaves.



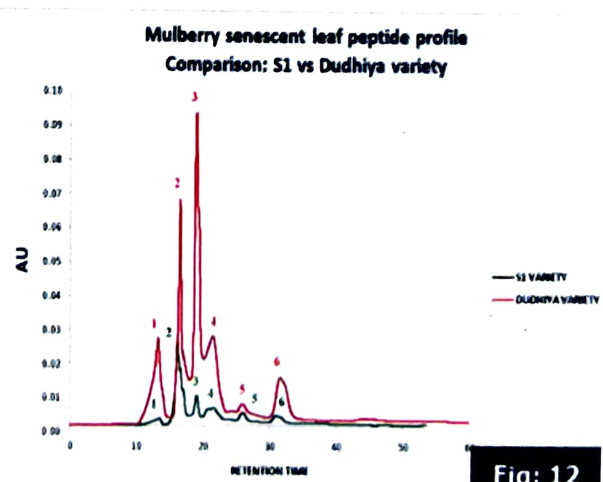


Figure 11: Comparative analysis of HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 and Dudhia mature leaves. Figure 12: Comparative analysis of HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 and Dudhia senescence leaves.

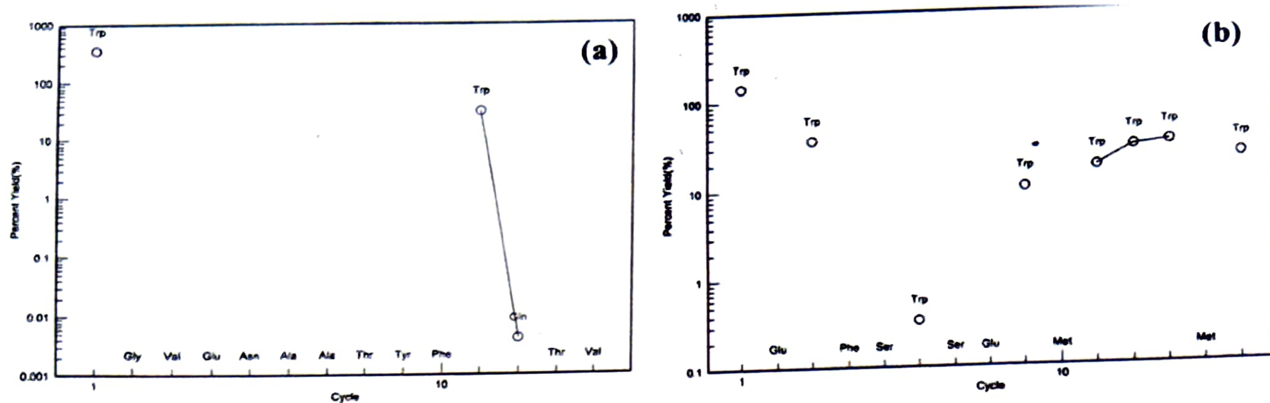


Figure 13: (a) and (b) shows the % of repetitive amino acid of S1 peptides and Dudhia respectively.

from S1 and Dudhia (Figure 11 and 12 respectively) were evaluated for determining the changes of HPLC peaks during maturation and senescence of mulberry leaves. At the time of maturation, non-overlapping peak 2 of HPLC of Dudhia genotype might be responsible for alteration of bioactivity, whereas during senescence, peptides isolated from Dudhia genotype comprising greater abundance might contribute inhibitory function as decoded from HPLC chromatogram.

The sequence of S1 mature peptides includes 14 amino acids which are "WGVENAATYFWQTV" with 100% reliability observed after 4th cycle of analysis in Try-His-Lys-Ala- followed by Ala-Try-Glu-Gly and Ala- Try-Pro-Asp as well as Try, Asp, Lys and Gly. On the other hand Dudhia mature peptides had 15 amino acids namely Trp-Glu-Trp-Phe-Ser-Trp-Ser-Glu-Trp-Met-Trp-Trp-Trp-Met-Trp with 87.7% reliability after 4th cycle. Figure 13 (a and b) shows the pattern of repetitive amino acids present in S1 and Dudhia mature peptides. From Figure 13 it was noted that Dudhia peptides contain repetitive units of tryptophan.

Conclusion

From our study it might be concluded that low molecular weight peptides especially at 0.5-3 k Da ranges could function as beneficial supplementary nutraceutical with mulberry leaves for silkworm rearing and could significantly improve economical attributes of rearing system. While Dudhia was refused by silkworm larvae, low molecular peptides isolated from mature leaves of Dudhia germplasm could influence larval growth. But the quantum of improvement of larval growth and economical attributes was comparatively lower than the bioactivity of S1 peptides. From this observation it can be stated that farmers would be potentially benefited if they use mulberry leaves treated with antioxidant enriched peptides as a food for silkworm rearing. However, more investigations will be required in this field for determining the exact mechanism of action.

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Integrated management of seedling blight disease of tea caused by *Sclerotium rolfsii*

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Abstract

Tea is one of the important plantation crops in Nepal. One of the important fungal pathogens *Sclerotium rolfsii*, causing seedling blight disease in tea was found to be predominant in the nursery grown plants. The art and science of plant disease control has moved in the direction of biological control of plant pathogen is a distinct possibility for the future and can be successfully exploited in modern agriculture, especially within the framework of integrated disease management systems. Effective integrated management practices against *S. rolfsii* were developed using neem cake, oil cake, aqueous leaf extract of *Azadirachta indica,* bio-control agent like *Trichoderma harzianum* and calixin (0.1%) *in vivo.* Combination with cow dung, neem cake, oil cake, chicken manure and rabbit manure, disease reduction were insignificant. However, combination with neem cake and oil cake showed 66.4% disease incidence, whereas in oil cake, neem cake and *Azadirachta indica* in combination disease incidence were recorded 11.1%. Under pot culture conditions *T. harzianum* alone and in combination with neem cake, oil cake and *Azadirachta indica* provided best effective management practices of seedling blight in all the three modes of application *viz.,* simultaneous, repeated and post infection.

Introduction

Tea is the important cash crop in Nepal. A number of fungal pathogens causes disease in tea plants. One of the important fungal pathogen is *Sclerotium rolfsii* which causes sclerotial blight in tea. A number of fungal pathogens cause diseases of tea which reduces the quality and quantity of tea production. Sclerotial blight caused by *Sclerotium rolfsii* Sacc. (telomorph: *Athelia rolfsii* (Curzi) Tu and Kimbrough = *Corticium rolfsii* Curzi) is one of the fungal diseases which appears in the nursery grown tea seedlings. Effective integrated management practices against *S. rolfsii* were tested *in vivo.* Integrated Disease Management (IDM) as applied to disease means using all the tactics available to the grower (cultural, biological, host plant resistance and chemical) that provides acceptable yield and quality at the least cost and is compatible with tenets of environmental stewardship. The art and science of plant disease control has moved in the direction of biological control of plant pathogens, including use of introduced antagonists. It is now widely recognized that biological control of plant pathogen is a distinct possibility for the future and can be successfully exploited in modern agriculture, especially within the framework of integrated disease management systems. Integrated control is a flexible, multi-dimensional approach to disease control utilizing a range of control components such as biological, cultural and chemical strategies needed to hold diseases below damaging economic threshold without damaging the agro-ecosystem. Effective integrated management practices against *S. rolfsii* were developed using neem cake, oil cake, aqueous leaf extract of *Azadirachta indica,* bio-control agent like *Trichoderma harzianum* and calixin (0.1%) *in vivo.* Combination with cow dung, neem cake, oil cake, chicken manure and rabbit manure, disease reduction were insignificant. However, combination with neem cake and oil cake showed 66.4% disease incidence, whereas in oil cake, neem cake and *Azadirachta indica* in combination disease incidence were recorded 11.1%. Under pot culture conditions *T. harzianum* alone and in combination with neem cake, oil cake and *Azadirachta indica* provided best effective management practices of seedling blight in all the three modes of application *viz.,* simultaneous, repeated and post infection.

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In the present investigation an attempt was made to develop an effective integrated management strategy against seedling blight of disease of tea.

Materials and methods

Fungal cultures

Virulent culture of *Sclerotium rolfsii* Sacc (*Corticium rolfsii* Curzi) was obtained from Immuno-Phytopathology Lab, Department of Botany, North Bengal University. This was originally isolated from Teen Ali-17/1/54 and after completion of Koch's postulate, the organism was identified by the Global Plant Clinic, Diagnostic and Advisory Service, CABI Bioscience UK and designated as Sr-1. Besides, two more isolates (Sr-2 and Sr-3) of *S. rolfsii* which were used in this investigation were isolated from infected tea roots of TV-25 and UP-8 respectively. Cultures of *Trichoderma harzianum* (biocontrol agent) was also obtained from the laboratory, mentioned above.

Inoculation technique

Inoculum preparation

Fungal pathogen

According to Chowdhury and Sinha (1995), sand maize meal medium was prepared in the ratio of 3:1 (sand : maize). In the prepared sand maize meal medium fungal pathogen (*S. rolfsii*) was inoculated and incubated at 28^{0}C for 7 days. The inoculum was mixed with sterile soil at the ratio of 1:8. Fungus soil mixture (100 gm) were mixed with the top soil of earthen pots containing tea seedlings and kept for development of disease reaction.

Biocontrol agents

Trichoderma species prepared in several media *viz.*, wheat bran media (wheat-bran: sand 1:1, and 25 ml of water for 150 g of inoculum in each polythene packet); Saw dust media (saw dust and water), tea waste media (tea waste and water). Media were autoclaved and inoculated as above.

Inoculation of healthy tea seedlings in pot

One year old tea seedlings were planted in earthen pots containing 1 kg soil and allowed to be established. Regular watering was done for two weeks and then 100 g of pathogen inoculum was added carefully in the rhizosphere of each plant. Disease assessment was done after 2-week- intervals and up to 45 days of inoculation.

Inducing agents and their application

In vivo test

Mature leaves (500 g) each of *Azadirachta indica* and *Catharanthus roseus* were harvested, washed thoroughly with running tap water, rinsed with distilled water, air dried and macerated separately homogenized in a electric blender. The leaf extract was filtered through double – layered muslin cloth and centrifuged at 10,000 g for 30 minutes. The supernatant was collected and filtered through Whatman No.1 filter paper. Each filtrate was further filter sterilized and preserved as stock (100%) solution aseptically in bottles at 5^{0}C for further use. Leaf extracts were diluted (1:10) with distilled water, drops of Tween-80 was mixed and sprayed on tea plants with the help of sprayer. The control plants were sprayed with distilled water mixed with Tween-80. Spray was done four times at 7-day intervals. Both treated and untreated plants were inoculated with *S. rolfsii* and disease assessment was made.

Mustard oil cakes and neem cakes were allowed to decompose separately for a week in a clay pot covered with polythene. After decomposition, 100 ml of decomposed oil cake solution was added in each tea seedlings pots. The pots were then inoculated with *S. rolfsii*. Untreated control was kept for comparison. Growth behaviour also observed up to two months. Organic additives (cow dung, rabbit manure and chicken manure), 100 gm of each were taken separately and mixed in 1 kg of soil. These soil mixtures were separately kept in each pot. Tea seedlings were planted in each pot containing different organic components. After one week, 100 gm of pathogen (*S. rolfsii*) inoculum was added in the rhizosphere of each

tea seedling.

Mass cultures of *T. harzianum and T. viride* were prepared on carrier medium comprising of wheat bran and sawdust (WBSD) in 3:1 ratio. Five hundred grams of the contents of carrier medium moistened with 20 percent (w/w) distilled water was filled in each bag. These polythene bags were sterilized at 15 lb pressure for 1 h for 2 consecutive days. Each polythene bag was then inoculated with 4-6 days old bits (0.3 cm) of pure culture either of *T. harzianum* and *T. viride* and incubated at 28±1^{0}C. During incubation, these bags were gently hand shaken to promote uniform sporulation over the carrier medium and to avoid clusters. Addition of biocontrol agents in soil was done 10 days prior to inoculation with *S. rolfsii.* 0.1% of calixin was sprayed with distilled water on tea plants. The control plants were sprayed with distilled water mixed with Tween-80. Spray was done four times at 7-day intervals. Both treated and untreated plants were inoculated with *S. rolfsii* and disease assessment was made.

Result

In vivo evaluation

Growth promotion in tea seedlings

Tea seedlings of two varieties (B-157 and TeenAli-17/1/57) were grown in soil amended with neem cake and oil cake separately. Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded after one-month interval and up to two months following the treatment of neem cake and oil cake and after inoculation with *S. rolfsii.* Results (Table 1) revealed that the growth of tea seedlings had been increased following amendment with neem and oil cakes than those treated plants inoculated with *S. rolfsii* in relation to untreated uninoculated tea seedlings as recorded after two months following treatment.

Similarly seedlings of three tea varieties (UP-3, B-157 and K-1/1) were grown in soil amended separately with cow dung, rabbit manure and chicken manure. Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded after one month interval up to two months following the treatment of organic components and after inoculation with *S. rolfsii.* It has been observed that the growth of tea seedlings had been increased in treated uninoculated than treated inoculated tea seedlings (Table 2). Among the three treatments with organic components, rabbit manure gave very good and healthy growth of tea seedlings than chicken manure and cow dung.

Under pot culture conditions *T. harzianum* alone and in combination with neem cake, oil

Table 1: Growth promotion in tea seedlings following soil amendment with neem cake and oil cake

Tea variety	One month				Two months			
	Healthy		Infected		Healthy		Infected	
	Increase in height (cm)	Increase no. of leaves	Increase in height (cm)	Increase no. of leaves	Increase in height (cm)	Increase no. of leaves	Increase in height cm	Increase no. of leaves
T17/1/54	2±.02	4±.04	0	2±.03	5±.03	6±.02	1±.01	2±.03
Untreated								
Treated	2±.01	3±.04	1±.01	0	2±.03	8±.04	2±.03	4±.02
Neem cake								
Oil cake	1±.02	3±.02	2.5±.1	2±.03	2±.01	4±.03	2±.01	3±.02
B-157	1±.01	3±.03	1±.02	0	4±.04	6±.03	1±.01	2±.03
Untreated								
Treated	2±.01	2±.03	1±.02	0	0	1±.01	2±.03	3±.01
Neem cake								
Oil cake	1±.04	4±.03	1.5±.1	0	2±.03	0	2±.02	1±.01

± Stand for standard deviation; Average of three replicates

Table 2: Growth promotion in tea seedlings by different organic components after inoculation with *Sclerotium rolfsii*

Tea variety	One month				Two months			
	Healthy		Infected		Healthy		Infected	
	Increase in				Increase in			
	height (cm)	no. of leaves	height (cm)	no. of leaves	height (cm)	no. of leaves	height (cm)	no. of leaves
UP-3 Untreated	2±.0	0	1±.02	0	3±04	0	1±.01	0
Treated Cow dung	6±.0	1±.02	3±.07	1±.04	4±.03	1±.02	1.5±.02	0
Rabbit manure	9±.0	0	6±.02	0	6±.03	1±.04	4±.02	0
Chicken manure	4±.0	1±.03	2±.01	0	3±.02	1±.06	2±.04	0
B-157 Untreated	1±.0	0	0	0	1±.02	2±.04	1±.02	0
Treated Cow dung	3±.0	1±.03	3±.04	1±.02	4±.05	1±.02	1±.03	1±.04
Rabbit manure	8±.0	1±.06	5±.02	0	5±.03	1±.06	2±.01	1±.03
Chicken manure	4±.0	0	2±.06	1±.03	4±.07	3±.08	2±.07	1±.04
K – 1/1 Untreated	2±.0	0	1±.05	0	2±.02	1±.02	0	0
Treated Cow dung	3±.0	0	1±.03	0	2±.05	0	0	0
Rabbit manure	9±.0	4±.02	7±.04	0	8±.03	0	2±.01	0
Chicken manure	6±.0	2±02	4±.03	0	3±.06	3±.04	2±.03	0

± Stand for standard deviation; Average of three replicates

Table 3: Effect of simultaneous treatments with biocontrol, fungicide, organic amendments and plant extract on development of seedling blight of tea following inoculation with *Sclerotium rolfsii*

Treatment	Disease incidence (%)	Disease control (%)
Trichoderma harzianum	0	100
Oil cake with Neem cake	66.4	33.6
Oil cake, Neem cake and *Azadirachta indica* (aqueous extract)	11.1	88.9
T. harzianum with *Azadirachta indica* (aqueous extract), oil cake and neem cake	0	100
Cow dung, Neem cake and Oil cake	44.6	55.4
Chicken manure, Neem cake and Oil cake	47.5	52.5
Rabbit manure, Neem cake and Oil cake	46.6	53.4
T. harzianum, Calixin (0.1%) and *Azadirachta indica* (aqueous extract)	0	100
Untreated Control	100	0

Table 4: Comparative efficacy of application of organic amendments and formulation against *Sclerotium rolfsii*

Treatment	Disease incidence (%)		
	Simultaneous	Repetitive	Post infection
Trichoderma harzianum	0	0	0
Oil cake, Neem cake and *Azadirachta indica* (aqueous extract)	15.8	0	44.6
T. harzianum, Azadirachta indica (aqueous extract) Oil Cake and Neem cake	0	0	0
Cowdung, Neem cake and Oil cake	40.6	30.5	77.7
Rabbit manure, Neem cake and Oil cake	46.3	33.0	85.8
Chicken manure, Neem cake and Oil cake	47.5	35.5	88.2
T. harzianum, Calixin (0.1%), *Azadirachta indica* (aqueous extract)	0	0	0
Untreated Control	100	100	100

cake and *Azadirachta indica* provided best effective management practices of seedlings blight in all the three modes of application *viz.*, simultaneous, repeated and pot infection. Combination with neem cake and oil cake showed 66.4% disease incidence where as in oil

cake, neem cake and *Azadirachta indica* in combination disease incidence were recorded 11.1%. But in combination with cow dung, neem cake, oil cake, chicken manure and rabbit manure, results were insignificant as shown in (Tables 3 and 4).

Discussion

In vivo trials demonstrated that *Trichoderma harzianum* alone as well as in combination with neem cake, oil cake, aqueous extract of *Azadirachta indica* and calixin (0.1%) provided a total control of sclerotial blight disease. Similar results were obtained by Sonali and Gupta (2004) when *T. viride* alone and in combination with neem oil, neem cake and deodar needles used in radial growth of *S. rolfsii* resulted in a total control of the disease. But repeated application of neem cake, oil cake with various combinations of cow dung, rabbit manure and chicken manure were found to be less significant. Finally it was observed that *T. harzianum* and in combination with neem cake, oil cake, neem extract and calixin (0.1%) were found most effective in reducing disease incidence on tea seedling plants *in vivo*.There are several reports on the management of disease by Integrated Disease Management (IDM). Management of chickpea root rot and collar rot against *S. rolfsii* by integration of biological and chemical seed treatment was reported by Tiwari and Mukhopadhyay (2003). They observed that application of carboxymethyl cellulose (CMC) with *G. virens* powder (10^9 spores per g) in combination with vitavax provided maximum protection (81.9%) to the crop against chickpea root rot and collar rot pathogens in glasshouse. Chickpea seeds treated with GV powder + CMC + vitavax significantly increased seedling emergence (47.9%); final plant stand (85.8%) and grain yield (79.7%) which was statistically at par with the treatment GV powder + vitavax and GV suspension + vitavax in a sick plot. Upamanyu *et al.*, (2002) reported the management of root rot and web blight caused by *Rhizoctonia solani*. They obtained that *T. viride* showed the maximum tolerance to carboxin, tebuconazole and carbendazim followed by *T. virens*, *T. harzianum* and *A. niger* when used in integrated disease management along with fungicides and oil cakes both under glass house and field conditions. Soil amendment (cotton cake) + *T. virens* and carboxin (ST), mustard cake + *T. virens* + tebuconazole and soil amendement (mustard cake) + carbendazim (ST) were found effective in containing the root rot under glass house conditions while soil amendment (mustard cake) + carbendazim (ST) + carbendazim (FS) were found highly effective in reducing pre- and post- emergence root rot and web blight. Severity was best contained by soil amendement (mustard cake) + carbendazim (ST+FS) followed by tebuconazole + *T. virens* (ST) + carbendazim (FS).

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