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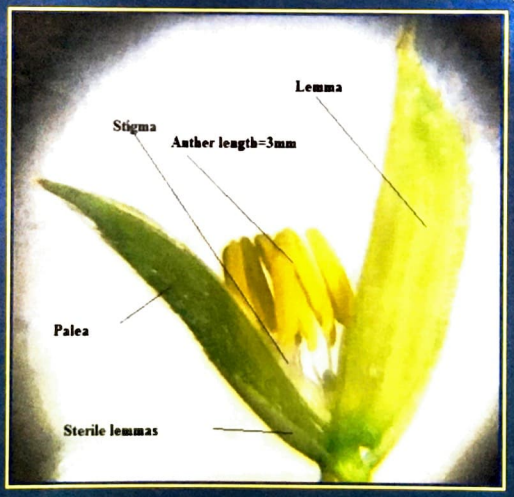

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Cover photograph: Single spikelet of Tulaipanji rice
(Photo Courtesy: Dr. SC Roy)

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Contents

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Association of AM fungi in the rhizosphere of *Thuja orientalis* (L)

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Abstract
Thuja orientalis (L) is used as an ornamental plant as well as medicinal plant in India from prehistoric times and instances were found in various manuscripts revealing its medicinal property against abdominal pain, hook worms, arthritis etc. AMF associated with rhizosphere of *Thuja* were extensively studied in relation to their population in soil, root-length colonization, histopathological study as well as their diversification. Predominant existence of various species of *Glomus, Gigaspora* were determined and their spore surface texture was examined using stereo microscope, besides, species of *Acaulospora, Scutellospora* etc. were also documented. Histopathological studies of host roots showed various types of hyphal network and arbuscules. AMF were tried to identify up to species level with the help of standard keys. The result indicated that the various spp. of AMF have established their colonization in host roots and the host plant have a significant role in root tissue colonization.

Key words: AM Fungi, *Gigaspora sp, Glomus sp, Thuja orientalis*

AM fungal associations have been described in ornamental maples (*Acer* sp.) (Brechet and le Tacon, 1984; Spiess *et al.*, 1991), Prunus genera, such as peaches (*Prunus persica* L.) (McGraw and Schenck, 1980) and sand cherry (Prunus ×cistena N.E. Hansen) (Morrison *et al.*, 1993), and have been recovered from roots of flowering dogwood (*Cornus florida* L.) (Sylvia, 1986). In India, only during the last decade the interest aroused on the role of VAM in rehabilitation of disturbed ecosystems (Kumar *et al.* 1999). Earlier studies on the occurrence of Arbuscular mycorrhizal fungi in medicinal plants mostly concentrate on rhizomes (Taber and Trappe 1982; Selvaraj *et al.* 1986). Later, Nasim (1990), Udea *et al.* (1992), Gautam and Sharma (1996), Selvaraj *et al.* (2001), Muthukumar *et al.* (2001), and Panwar and Tarafdar (2006) reported the occurrence of medicinal plants from India. However AMF association with some important medicinal plants of Sagar, Goa was performed (Deepak Vyas, 2007). Beside this AM association in various medicinal plants were extensively done in Indian subcontinent (Muthukumar and Udaiyan, 2001).

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Mycorrhiza form symbiotic associations between most of the terrestrial plants and play an essential role in plant growth, plant protection, survival, disease suppression and soil quality. The hyphal network/arbuscules promotes the bi-directional nutrient movement where soil nutrients and water move to the plant and plant photosynthates flow to the fungal network. AMF are obligate symbionts and improve the uptake of phosphate and other nutrients from the soil which results for drought and disease resistance to the host plant. The main benefit to the host plant in the mycorrhizal symbiosis is the enhanced uptake of immobile soil nutrients, in particular phosphorus (Jakobsen, 1999). Arbuscular mycorrhizal associations increase nitrogen accumulation in plant tissues as a result of the hyphae out competing for mineralized organic soil nitrogen (Ibijbijen *et al.*, 1996). *Thuja orientalis* (L) of cupressaceae family is an evergreen plant and is mostly used as ornamental plants in the gardens besides this plant is also known for its medicinal values where the leaves of these plants are used as antibacterial, antipyretic, antitussive, astringent, diuretic, expectorant and stomach upsets. Their use is said to improve the growth of hair. They are used internally in the treatment of coughs, hemorrhages, bronchitis,

asthma, skin infections, mumps, bacterial dysentery, arthritic pain and premature baldness. The leaves were harvested for use as required and can be used fresh or dried. Some proponents claim that thuja decreases the toxic effects of chemotherapy and radiation therapy. Herbalists prescribe thuja to treat viral and bacterial infections and coughs and other respiratory ailments, including strep throat and respiratory distress related to congestive heart failure. Herbalists also use it as a diuretic to increase urination, and as an astringent to "purify the blood," reduce inflammation, and cleanse the body of "toxins." Thuja is sometimes used with antibiotics to treat bacterial skin infections and herpes sores. It has even been used by some practitioners to induce abortions. Thuja ointment is applied to the skin for ailments such as psoriasis, eczema, vaginal infections, warts, muscle aches, and rheumatism. The association of AM fungi in such type of plants may vary greatly and is of much importance to know the details of the symbiosis. In the present investigation attempts have been made to find out the association of AM fungi in the rhizosphere of *Thuja orientalis* (L), since no such work has yet been reported.

Materials and Methods

Wet sieving and decanting method of Gerdemann and Nicolson, (1963) was used for the isolation of AMF spores. Approximately 250 gm of soil was suspended in 1 liter of water. Heavier particles were allowed to settle for a few minutes and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200 where BS stands for British Standard) are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension was then passed through these sieves resulting in trapping of heavier particles in the upper zone and light and smaller particles are trapped gradually in the sieves arranged in a descending order from fine to finer. The suspended water were allowed to settle for a 10 minutes and then the liquid was decanted again through the sieve and spores were collected by fine brushes and were kept in different petri plates according to their size and colors. Moreover for further observation for the purification of AMF spores sucrose gradient centrifugation method of Daniels and Skipper, 1982 was used. Spores and minimal amount of organic particles were further purified by suspending sieving in the 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) were poured through the sieve of 200 mesh and rinse with distilled water to remove sucrose residue. With the help of a simple microscope (20X) parasitized spores, plant debris etc were separated and clean spores were stained with Melzar's reagent and studied microscopically. For further use the spores were stored either in distilled water or in Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in 1 liter of boiled distilled water) at 4°C.

For the histopathological study, the root specimens were processed according to the method of Phillips and Hayman (1970). The root samples were cut into 1 cm and were cleared by 2% KOH/NaOH for 15 min at 90°C. Then the samples were washed thrice in tap water and kept for 30 min in 2% HCl. The root samples were boiled for 15 min with 0.05 Cotton Blue. Degree of contrast between fungal tissues and back ground plant cells was obtained according to the duration of storage of the tissues. 1% HCl was added to acidify the tissues as most histological stains are acidic.

The method of Phillips and Hayman (1970) which was followed for histopathological studies revealed that due to boiling in water bath twice, the extra radical hyphae, auxiliary cells and other fungal structures are dissociated in the solution resulting a wrong interpretation for the determination of the percent colonization. So this method was slightly amended and instead of boiling, the root samples were kept in 2% KOH for 72 hours and after washing by water and 2% HCl, the root samples were again kept for 72 hours in 0.05 cotton blue and lactoglycerol. The total treatment was done seldom disturbing the root fragments, keeping in petri-plates which retain the extra radical hyphal structures along with auxiliary cells, intact spores, vesicles etc.

Results and Discussion

AMF spores were collected from the host plant of three different sites. Morphological features of isolated AMF spores were critically examined with special reference to variation in size, wall thickness, shape, wall layers viz. germinal wall, coriaceous wall, amorphous wall, beaded wall etc. In every aspect the genus *Glomus* was predominant followed by *Gigaspora, Acaulospora, Scutellospora and Sclerocystis*. Fungal species identification were carried out using valid and standard keys of Schenck and Perez (1990) and Trappe (1982)

Percent root colonization with AMF was determined by counting all the infected and uninfected segments of root tissues and the percentage of infection was calculated as: AMF infection (%) = (infected / total fragments taken (no.) = 26 (average of five readings) / 30 (Total no. root fragments taken) x 100 = 0.86 x 100 = 86.66%. Simultaneously, analysis of soil samples collected from the rhizosphere of Thuja plants were done in order to find out the physico-chemical properties of soil where association of the AMF prevails. Results have been presented in Table-1.

Table 1. Rhizosphere soil analysis of *Thuja orientalis* (L)

Soil type	Sandy-clay	P_2O_5(ppm)	38.88
Sand(%)	55	K_2O(ppm)	96.46
Silt(%)	02	Organic Carbon(%)	1.29
Clay(%)	41	Nitrogen(%)	0.18
pH	5.01	Spore No./100gm of soil	84
Moisture(%)	13.68	Colonization(%)	86.66

Association of AMF in rhizosphere of *Thuja orientalis*

Thorough investigation on the association of AMF in the rhizosphere of Thuja plants were done considering twenty different soil samples collected from various location of North Bengal. Population percentage of dominant mycorrhizal fungi e.g. *Glomus*, *Gigaspora*, *Acaulospora* and *Scutellospora* has been determined and presented in Figure 1.

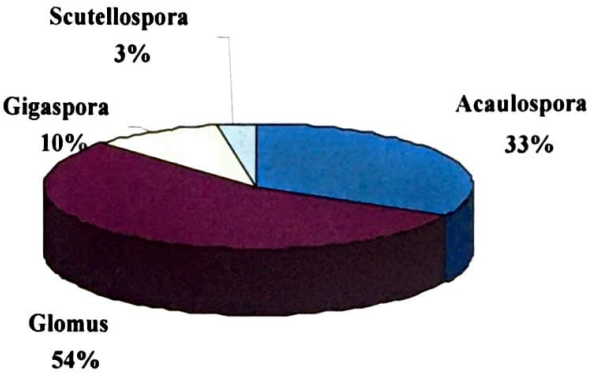

Figure 1. Population percentage of AMF spores in *Thuja orientalis*

Detail microscopic observations were made, photographs taken, all the essential spore characters were noted and compared with the available monographs and other literature. Description of the spore characters of prevalent AMF are enlisted below.

Acaulospora bireticulata

Spores are globose and brownish in colour, diameter ranges from 280-410µm. Surface ornamentation was prominent. Spore wall consists of three layers. Spores were borne laterally from the neck of a sporiferous saccule (Fig.2. I)

Acaulospora capsicula

Spores were bright red or orange red in colour, globose to subglobose with three distinct layers. Layer 1, forming the spore surface, evanescent, hyaline, 1.5µm thick in average and continuous with the wall of a sporiferous saccule, usually completely sloughed in mature spores. Layer 2 laminate, smooth, orange red to capsicum red 4.2µm thick. Layer 3 laminate, rigid, hyaline, 2.4µm thick, easily separating from layer 2.

Glomus fasciculatum

Colour pale yellow to bright brown with globose to subglobose in shape. Spores were produced directly with one or more subtending hyphae attached to it. Spore wall was continuous. Spore wall consisting of three layers (L1, L2, and L3). Spore size ranges from 70-120µm in diameter (Fig.2 B).

Glomus aggregatum

Spores globose to oval in shape. Size ranges from 40-120µm in diameter, color-pale yellow. Formed

singly or in sporocarps. Spore wall consist of 1-2 layers. Sporocarps are formed in loose clusters, from a single stalk, diameter ranges from 200-1800 x 200-1400µm in size.

Glomus mosseae

Brown to orange-brown in colour, shape, globose to sub-globose with an average diameter of 200µm. Presence of three hyaline layers with subtending hyphae attached. Hyphae was double layered (Fig. 2.A).

Glomus drummondii

Spores occur singly in the soil; develops from the tip of extra radical hyphae of mycorrhizal roots. Spores were golden yellow, globose to subglobose, average diameter is 70µm in diameter; single subtending hypha attached with the spore. Spore wall consists of three distinct layers (Fig.2 C).

Glomus constrictum

Spores were single in the soil with one subtending hypha, colour brownish orange to dark brown globose to subglobose; 160µm diam in average. Spores consists of one wall containing two layers, most juvenile spores with spore wall layer 1 only. Subtending hypha brownish orange to dark brown; straight or curved; usually markedly constricted at the spore base, sometimes cylindrical, flared to funnel-shaped; composed of two layers continuous with spore wall layers 1 and 2. *Glomus constrictum* is one of the most frequently found arbuscular mycorrhizal fungus in cultivated and uncultivated soils (Fig.2 D).

Glomus clarum

Spores, single in the soil; hyaline to pale yellow, globose to subglobose; 150µm diam; sometimes ovoid; 90-100 x 140-180µm; with one subtending hypha. hyaline to pale yellow straight to curved; wall of subtending hypha hyaline to pale yellow, thick at the spore base; composed of three layers (Fig.2 E).

Glomus aggregatum

Spores formed singly in the soil, in aggregates, in roots, aggregates ranges from 160-1600 x 250-1900µm, without a peridium, with two to over one hundred spores loosely distributed. Colour of spores were pastel yellow to yellowish brown; mostly globose to subglobose; rarely pyriform to irregular; usually with a single subtending hypha, rarely with two (Fig.2 G).

Gigaspora gigantea

Spores single in the soil; formed terminally or laterally on a bulbous sporogenous cell; greenish yellow (globose to subglobose; 300µm diam; sometimes ovoid; 250 x 270µm. Subcellular structure of spores consists of a spore wall with two layers and one germinal wall (Fig.2 H).

Gigaspora margarita

Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell. Spores yellowish white to sunflower yellow; globose to subglobose; 357µm diam; sometimes ovoid; 320 x 370µm. Sporogenous cell orange to brownish yellow. Structure of sporogenous cell composed of two layers. Layer 1 hyaline, 1.7µm thick approximately. Continuous with spore wall layer 1. Layer 2 orange to brownish yellow, 5.6µm thick, continuous with spore wall layer 2.

Scutellospora pellucida

Spores single in the soil; formed terminally on a bulbous subtending hypha; hyaline to yolk yellow; globose to subglobose; 195µm diam; sometimes ovoid; 130-155 x 160-235µm.

Scutellospora rubra

Spores colour: dark orange-brown to red-brown at maturity, immature spores are white to cream with a rose tint under a dissecting microscope. Shape: globose to subglobose. Size 180µm in average (Fig.2 H).

Besides, frequency of identified AMF in three locations were also critically analysed. The results have been computed and presented in Table- 2.

The major biological characteristic of AMF is their obligate biotrophic nature. This means that each of their life cycle steps requires the association with a living plant. As with most of the filamentous fungi, AMF propagation can occur either by spores differentiation and germination or by mycelium extension through soil and roots. Spores are differentiated by budding intercalary or apically on

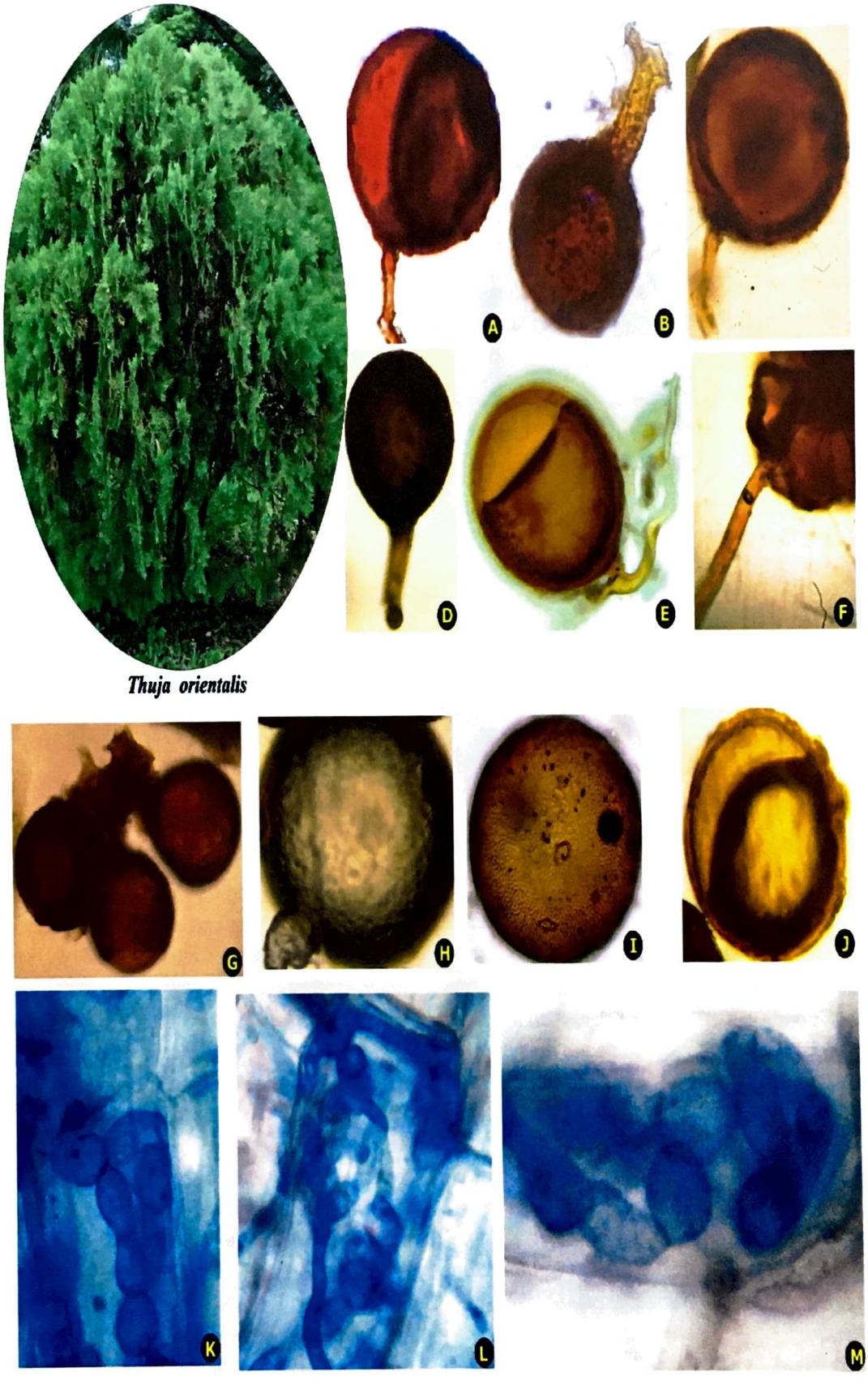

Figure 2. A - M. Spores and Histopathology. A. *Glomus mosseae*; B. *Glomus fasciculatum*; C. *Glomus drummondii*; D. *Glomus constrictum*; E. *Glomus clarum*; F. Sporocarp of *Glomus* sp. G. *Glomus aggregatum* H. *Gigaspora gigantea* I. *Acaulospora bireticulata* H. *Scutelospora rubra* K.M Different types of arbuscules formed by *Gigaspora* spp.

Table 2. The frequency of AMF spores in the study areas

Name of AMF	LOCATION		
	Darjeeling 27°03'N 88°18'E	Coochbehar 26°22'N 89°29'E	Jalpaiguri 26°32'N 88°46'E
Acaulospora			
A. alpina	+	+	+
A.bireticulata	+	+	+
A.capsicula	-	+	+
A.delicata	-	-	+
Glomus			
G.mosseae	+	+	+
G. fasciculatum	+	+	+
G. aggregatum	-	+	+
G. albidum	-	-	+
G. ambisporum	+	+	+
G. constrictum	+	+	+
G. badium	+	+	+
G. deserticola	+	+	+
G. drummondii	+	+	+
Gigaspora			
Gi. gigantea	+	+	+
Gi. margarita	+	+	+
Gi. albida	-	+	+
Scutellospora			
S. calospora	+	+	+
S. pellucida	+	+	+
S. rubra	-	+	+

hyphae. AMF species identification is based on spore characters, spore wall architecture, and the morphology of subtending hyphae. Some molecular tools to differentiate among AMF species and strains have been developed. Sexual reproduction has not yet been observed for these symbiotic fungi; therefore they are considered asexual. Fungal filaments grow through soil particles and come in contact with young plant roots, the fungal threads its way through root surface, and then grow between and inside cortical cells. The wide dispersal of the fungl network through its filaments gives the plant-root mycorrhiza access to a much larger volume of soil than the root system itself. The establishment of mycorrhizal networks in roots and soil constitute a soil-root fungal continium, which is required for beneficial symbiotic exchanges between fungi and plant. AM mycelium can spread throughout the soil surrounding the root system and increase the ability to explore soil areas, accessing water and nutrients for plant roots. Benefits to plants are improved by water and nutrient uptake, enhanced P transport, drought and disease resistance. Benefits to fungi are the supply of photosynthates to the fungal network located in the cortical cells of the plant and the surrounding soil. All water, nutrients, and photosynthates exchanges occur via the fungal filament network that bridged plant rhizosphere and plant roots. Arbuscules found in the roots of *Thuja* are haustoria-like structures that are formed by profuse dichotomous hyphae branching after penetration into inner plant cortical cell walls, forming an interface. These arbuscules are the exchange site of nutrients. In *Thuja* the arbuscules

formed are highly coiled with swollen trunks and is formed either singly or in clusters and vesicles are absent nearer to the arbuscules which indicate that these arbuscules are formed by *Gigaspora* sp. In some root fragments deep blue in coloured, thin walled, ellipsoidal structures were found in abundant were known as vesicles. Auxiliary cells were formed by short ramifications occurring at one or simultaneously at both sides of extra radical hyphae. High population of AM fungi such as species of *Glomus, Gigaspora, Scutellospora* and *Acaulospora* were obtained from the rhizosphere soil of *Thuja.*

Acknowledgements

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Assessment of Genetic Diversity in F2 Rice Seed Population of a Cross between Tulaipanji and Ranjit Using Morphological, Physicochemical and SSR Markers

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Abstract

Tulaipanji rice (*Oryza sativa* L.) variety is a region specific traditional cultivar of North Dinajpur district (West Bengal). It is low yielding (1.8 t/h) cultivar due to their poor harvest index and other genetic factors such as tendency to lodging and susceptibility to foliar diseases such as blast and bacterial blight, tungro virus etc. Tulaipanji is non-Basmati aromatic rice and people prefer it due to its soft fluffy grain quality with mild fragrance and easy to digest. Hence it has high demand in the market and costs Rs. 80/kg. Demand gap can be fulfilled by developing a new improved variety of Tulaipanji combining its grain quality attributes with high yield potential genes/QTLs and resistance to diseases. Hybridization was made (during kharif season 2011) between Tulaipanji and Ranjit (HYV) for the introgression of short stature, high yielding responsible genes/QTLs from Ranjit to Tulaipanji for its improvement. F_2 seeds population were collected and analysed based on morphological and physicochemical parameters for their genetic variability and screening. Genetic diversity among the F1 plants and parental lines were analysed using microsatellite based SSR primer in PCR amplification. SSR markers in relation to disease resistance gene/QTL such as Xa21, Pita440 and Pib sub 3-5 were used to screen the breeding lines. Xa21specific amplified band was observed in cultivar Ranjit (HYV) which was located on chromosome 6 (resistance gene/QTL for bacterial blight) and accordingly introgressed into one of the line of F1 plants (Tulaipanji x Ranjit). There was no gene/QTL specific band was amplified for SSR marker of Pi440 and Pib sub 3-5 in the parental or F1 lines for blast resistance. Grain quality QTL specific markers such as Waxy, Sbe 2 and RM225 were employed to screen the F1 and parental lines for apparent amylose content (ACC). Apparent amylose content (ACC) was 22% in Tulaipanji and 25% in Ranjit and F2 seed showed in between 22 and 25% amylose. Breeding lines showing desired traits in relation to disease resistance and quality traits can be used for the development of improved Tulaipanji rice of North Dinajpur through marker assisted selection (MAS) using SSR markers.

Keywords: Tulaipanji rice, F2 seeds, SSR markers, Xa21, Waxy gene, Amylose content.

Introduction:

Cultivated rice belongs to two species- *Oryza sativa* (L.) and *Oryza glaberrima* (Steud.). *Oryza glaberrima,* the African cultivated rice, is grown on a limited scale in West Africa. *Oryza sativa* has two subspecies- *O. sativa* sp indica and *O. sativa* sp japonica. The subspecies japonica is mainly cultivated in Japan, Korea, Taiwan and Italy, and their genetic diversity is narrow. On the other hand, the subspecies indica is mainly cultivated in China, India, Thailand, Indonesia, and their genetic diversity is wide. About 90% of the cultivated rice belongs to the indica type and not japonica.

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Cultivated rice *Oryza sativa* (L.) subspecies indica is grown worldwide especially in Asian subcontinent. Rice is grown in a wide range of agro-ecological conditions in India. Based on the harvested area of 45 million ha (Mha), about 55% of the crop is planted in Eastern India, 21% in North, 18% in the South and about 6% in West. Rice is central to the food security of over half the world's population – it is vital for the nutrition of much of the population in Asia, as well as in Latin America, the Caribbean and in Africa, totalling over 3.5 billion people worldwide that eat it every day, and depend on it for more than 30-40% of their daily calories. Asia can be considered as *'Rice Basket'* of the world because where 90 per cent of world's rice is

grown. Maximum rice (90%) are grown in Asia as well as consumed with 60% of population and where about two-thirds of world's poor live (Khush and Virk, 2000). Hence, *'Rice is life'* in Asian subcontinent. Only 4-5 per cent of world rice production enters the Global market. That means, any shortfall in rice production in the major rice growing countries could be disaster for food security. Rice occupies pivotal role in Indian agriculture. It is the staple food for more than 70% Indians and source of livelihood for 120-150 million rural households. It contributes 43% to the total food grain and 53 per cent to the cereal production and thus holds the key to sustain food sufficiency in the country (Siddiq *et al.*, 2004). To feed 5 billion rice consumers in 2025, we have to develop rice varieties with high yield potential and greater yield stability (1.1% yield increase every year) with quality grains for food sufficiency. To keep rice prices stable and affordable, IRRI estimates that an additional 8-10 million tons of rice needs to be produced every year. The population of rice consumers is increasing at the rate of 1.8% annually. The present annual rice production of 700 million tons must be increased to 850 million tons by 2025. There are no additional lands available for rice cultivation. In fact, the area planted to rice is going down in several countries due to pressures of urbanization. Thus, we need the rice varieties with higher yield potential and yield stability for meeting the challenges of increased rice production. During this intensive breeding effort, varieties have been developed which have genes from various ecotypes of rice. Even the genes from wild species have been introduced into modern varieties. Thus, ecotypic differentiation present in the landraces of rice no longer exists in the improved varieties. Environmentalists have expressed concern about the reduction in biodiversity due to replacement of numerous old varieties by a selected number of improved varieties. However, genes of numerous landraces have been incorporated into the new varieties. For example, IR64 has 20 landraces in its ancestry. Moreover, most of the traditional varieties grown before the green revolution have been preserved in the gene banks and are available for future rice improvement (Khush, 1997).

A rapid rise in world population and decreasing agricultural land exert pressure on the production and productivity of rice cultivars (Khush, 2005). Currently India produces 104 million tonnes of rice equal to 43% of the total grains from 45 million hectares of land out of 297 million hectares total agriculture land. We need approximately 150 million tonnes of rice by 2030 to feed 1.378 billion Indian people (Goyal and Singh 2002). Increasing world population, shrinking cultivable rice land area, water scarcity and excess, evolution of new biotypes of pests and diseases, deteriorating soil health and climate change pose major cause of concern to rice breeders to increase production and productivity with multiple resistances to biotic and abiotic stresses which causes low yield and quality (average yield 2.1 t/ha in compare to world yield 4.1 t/ha). Thus there is a plateau in yield levels of existing rice varieties. Different breeding strategies such as introduction, selection, recombination breeding, heterosis breeding etc were practiced during and after green revolution to increase the yield levels of rice. Although, yield was improved using these breeding strategies, the yield levels have stagnated subsequently.

Not only the biotic/abiotic stress problems but also narrow genetic diversity in modern cultivars poses major constraint to further increases in productivity. Molecular markers provide opportunities to map resistance genes and accelerate the application of marker assisted backcross (MAB) breeding through the precise transfer of target genomic regions into the recurrent parent (Jena and Mackill, 2008; Lewis and Kernodle, 2009). Backcross breeding is often used in a conventional breeding program to transfer specific genes into elite cultivars since long back (Allard, 1961). The basis of MAB breeding is to transfer a specific gene/allele of the donor parent into the recurrent parent genome while selecting against donor introgressions across the rest of the genome. The effectiveness of MAB breeding depends on the availability of closely linked DNA markers for the target locus, the size of the population, the number of backcrosses and the position and number of markers for background selection (Frisch and Melchinger, 2005). In addition, molecular markers precisely estimate the

introgression of chromosome segments from donor parents and can speed up the recipient genome recovery via background selection (Suh *et al.*, 2009). Recent advances in rice genomics research (IRGSP, 2005) has made it possible to identify and map precisely a number of genes through linkage to DNA markers. Some important markers are tightly linked to tolerance/resistance to blast, bacterial blight, virus diseases, brown plant hopper, drought, submergence, salinity, low temperature and improved agronomic and grain quality traits (http://www.gramene.org). Historically, many breeding programs took yield potential as the primary target. As a result many popular high-yielding cultivars and hybrids have relatively poor quality. But there is also a tremendous need for improvement of rice grain quality because it has market demand due to increased living standard. So, the improvement in cooking, eating, and appearance quality of the rice grain has become a priority area in rice breeding programme (Zhang, 2007). So, rice varieties with increased yield potential in adverse conditions as well as in normal conditions have to be developed in order to sustain the rice production across wide range of environments and over the years. The challenge is to produce these additional quantities with less land, less water, and less labor, in more efficient, environmentally-friendly production systems that are more resilient to climate change. Earlier, wild and wild relatives were frequently used in resistance breeding programs to improve simple traits such as resistance to various pests and diseases. They were rarely used to improve complex traits such as yield, drought tolerance, salinity tolerance etc. Currently, advanced backcross Quantitative Trait Locus method followed by molecular mapping studies showed that phenotypically poor wild species can contribute genes for improving yield and such loci can be mapped and introgressed in to elite cultivars (Gur and Zamir, 2004). This enables the efficient use of wild and wild relatives to broaden the genetic base of the existing cultivars and also to improve complex traits by marker aided introgression of superior wild alleles (Laxuman *et al.*, 2011).

Tulaipanji is a non-Basmati aromatic scented rice of West Bengal and specifically cultivated in the district of North Dinajpur, West Bengal and sold at the rate of Rs.70-80/kg and it has high demand in the rice market of West Bengal and other states.

Total cultivated land of North Dinajpur is 2,41,292 hectare, out of this kharif aman rice area is 1,82,975 hectare in 2012. District has two Subdivisions- Islampur and Raiganj. Islampur subdivision having 1,04,950 hectare agricultural land in 5 block and Raiganj Subdivision having 78025 hectare in 4 block which are under Aman rice cultivation. Raiganj Block has total cultivated land area 35,200 hectare, out of which 29,950 hectare is used for Aman rice in 2012 and out of Aman area 2120 hectare is used for Tulaipanji cultivation (which is 14.97% land of this block). Total area under Tulaipanji rice cultivation in North Dinajpur is: 4485 hectare in 2012 (2.45% in respect to other aman rice in the District) and yield is 1.8 t/h on an average. Main problem facing by the farmers: Main constrains of the cultivation of this high demand aromatic rice (Tulaipanji) is susceptible to various diseases and pest and moreover low yielder (1.8 t/h). In spite of low yield and disease susceptibility farmers are still cultivating this Tulaipanji for high price in the market and to conserve their inherited agricultural practices and cultural heritage.

Germplasm diversity is the mainstay for crop improvement and genetic dissection of complex traits. Due to aggressive introduction of modern high yielding varieties (HYV) in the region has resulted in the loss of a large number of landraces especially from irrigated lands, which leads to narrowing down the gene pool of the rice diversity. The present investigation looks at the genetic variability and specific morpho-quality traits to conserve rice germplasms on farm and to maximise its use in rice breeding. Wide genetic resources may be required to either increase the genepool for germplasm improvement or for the development of new cultivated varieties. Accurate assessment of the levels and patterns of genetic diversity can be invaluable in crop breeding for diverse applications including introgression desirable genes from diverse germplasm into the available genetic base. Conservation of the rice gene pool is necessary so that breeders can get access of these genetic resources for future use and for widening

the narrow genetic base of the developed varieties.

Yield attributing characters are complex and controlled by many physio-morphological traits which are independently governed by polygenes and are much influenced by environmental factors. Although conventional approach was used to address the genetic improvement of yield and yield attributing traits and its stability. But they show low heritability in the field and expensive in attaining progress (Nguyen *et al.*, 1997). Alternatively molecular breeding may achieve the target specificity at faster rate in compared to conventional approach. Use of marker assisted breeding (MAB) would offer opportunity in identifying specific genes through QTLs for complex traits combining yield and yield related traits in crop plants (O'Toole, 1989). Marker assisted selection (MAS) of genes for pyramiding bacterial blight resistance genes, Xa13 and Xa21 (Mahapatra *et al.*, 2006) and QTLs controlling root traits (Steele *et al.* 2006) in elite backgrounds are example for success stories of molecular breeding in rice improvement. The introgression of useful traits from diverse donors into elite background by back crossing to develop introgressed lines (BILs) is the best approach for QTL mapping as well as genetic improvement of elite cultivars (Li, 1999). It would allow the simultaneous identification of desirable alleles of many QTLs and generation of backcross inbred lines (BILs) with incorporated QTLs for evaluation in target environments.

After reviewing the literatures and considering the origin of the problem, the present investigation will be made first time to evaluate the pattern and extent of genetic diversity exists in Tulaipanji rice employing important morpho-quality, physico-chemical and agronomic traits and introgression of yield and disease resistance traits using Ranjit (HYV) as donor parent. In the present study F2 seeds population is used as mapping population for screening the breeding lines with desired traits and targeting to broaden the gene pool for genetic improvement of Tulaipanji rice variety using marker assisted selection (MAS) in molecular breeding.

Materials and Methods

Experimental site

The present investigation was carried out during kharif 2011 and 2012 at Experimental Rice Field, Department of Botany, University of North Bengal, WB, India, representing the low land with sandy-loam soil, acidic pH which is located at latitude of 26° 84A North and longitude of 88° 44A East.

Experimental material

The experimental material for the present investigation comprised of Tulaipanji rice (*Oryza sativa* L.) variety of North Dinajpur district as recurrent parent and Ranjit as donor parent. Ranjit is a high yielding variety (HYV) has genealogy of Pankaj and Mashuri (including Taichung 65 and Tangkai rotan).

Experimental Layout

Hybridization was performed according to IRRI protocol between Tulaipanji and Ranjit during kharif 2011 for introgression of Yield/quality/disease resistance gene/QTLs in recurrent parent through marker assisted selection. The introgressed population is genotyped and mapped with six well distributed SSR markers selected from the linkage map developed at Cornell University (Temnykh *et al.*, 2001) and http://www.gramene.org for the following trait specific gene/QTL markers- Waxy, Sbe2, RM225, Xa21, Pita 440 and Pib sub 3-5. The mapping population of F2 seeds are evaluated along with parental lines for morphological and physicochemical analyses. The mapping population is evaluated with two replications in randomized complete block design during kharif season (2012). Each line was planted in two rows of 2m length with row spacing of 20cm and 10cm between plants. The recommended package of practice is followed for raising a good and healthy crop.

Procedure of artificial cross-pollination

The rice inflorescence is a panicle bearing singled-flowered spikelets. The flower is surrounded by a lemma and palea, structures that form the hull or husk that enclose the caryopsis. The outer glumes are sterile and usually obscure. The blooming of rice normally occurs between 10 am and 2 pm. Pollen is generally shed at the time the flower opens

with blooming of the spikelet, starting at the apex of the panicle and proceeding downward. Rice flowers are emasculated in preparation for crossing by cutting the tip of the floret and removing the anthers. (Fig. 7) About one-third to one-half of each

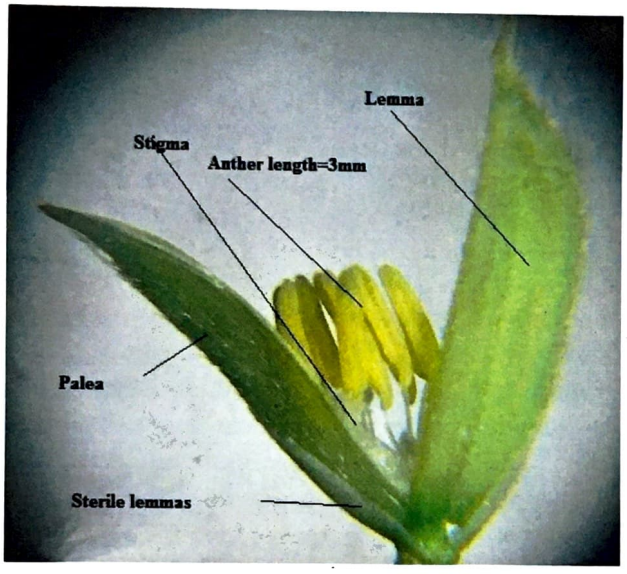

Figure 7: Rice flower showing anther and stigma and other parts of the spikelet of cultivar Tulaipanji.

floret is cut off at a slant to expose the anthers. Anthers are removed with forceps. Emasculations performed in the morning may risk anthers opening during their removal, and so it is safer to remove them in the afternoon (when anthesis has ceased for the day at 3 pm). After emasculation, flowers are covered with paper bag to protect them from natural cross-pollination until they open and are ready for pollination. The rice panicles are pollinated in the morning following emasculation. The male panicles are cut and flag leaf removed. These panicles are watched closely for anther extrusion, and used thereafter for pollination. The paper bag is taken out from the female and the pollen shaken over the emasculated panicle. After pollination, the panicles are covered by the same paper bag for protection from stray pollen and tagged properly. Pollen of rice is short-lived, only remaining viable for about 5 minutes. After 25 days of pollination F1 seeds are collected from the parent plant (October 2011) and labelled accordingly (cross I: Tulaipanji x Ranjit and cross II: Ranjit x Tulaipanji). F1 seeds along with lines were germinated on earthen pot with soil mixture in next year kharif season (2012) for further analysis. Seedlings were transplanted after 21 days of germination and maintained with proper agronomical practices for good crop. F2 seeds were analysed for grain morphology, quality and physicochemical analysis. F3 seed population will be collected from this kharif crop during October, 2013.

Recording of observation

The data on morphological, physicochemical and other traits are recorded from three randomly selected representative plants in all the genotypes in each replication. The standard method (DUS-Distinctness over Uniformity and Stability, Govt. of India) is used for recording observation for each of the character which includes the following- plant height, stem thickness, penultimate leaf length, penultimate leaf breadth, flag leaf length, flag leaf breadth, panicle length, panicle branching, seed/ panicle, grain weight.

Phenotyping for grain quality traits

The grain quality was measured according to IRRI protocol (Juliano and Villareal 1993)- grain length (GL), grain breadth (GB), awn length, kernel length (KL), kernel breadth (KB), ratio of GL/GB, ratio of KL/KB, cooked kernel length (CKL), cooked kernel breadth (CKB), ratio of CKL/CKB, linear elongation ratio, cooking time etc. In this present investigation other quality parameters such as aroma, gelatinous temperature (GT) and alkali spreading value (ASV) were also considered. These quality traits was represented through distinct number only (0, 1, 2, 3 etc) to denote their magnitude. The alkali spreading value (ASV) was determined by the method of Little *et al.* (1958) with minor modifications. A set of five polished rice grains from each line was immersed in a freshly prepared 1.7% KOH solution and incubated at 30 f C for 23 h and spreading of the rice grains was recorded by visual observation (Fig. 4) in seven categories from 1(unaffected) to 7 (completely dissolved). The aroma of polished rice grains was determined by a sensory evaluation protocol according to the method of Sood and Siddiq (1978) with minor modification. Ten milled rice grains were placed in a 50 mm Petri plate containing 10 ml of 1.7% KOH and incubated at room temperature for 10 min with

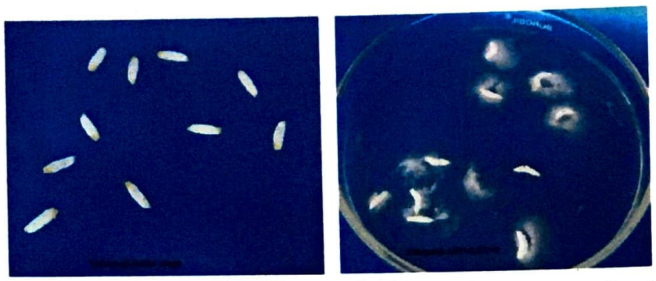

Figure 4. Alkali spreading value (ASV) was determined in Tulaipanji for quality assessment.

lids on. The lids were then opened one by one and samples were smelled and rated for aroma by sensory evaluation in a scale of 0-3, where 0 was non-aromatic and 3 was highly aromatic. Two blind checks, Pusa Basmati 1(moderately aromatic) and Ranjit (non aromatic) were included in this observation.

Amylose measurements by Spectrophotometer

Starch is a major component (90-93%) of rice seeds. Starch is composed of a mixture of two forms of glucose polymer, amylose and amylopectin. Amylose is principally a linear polymer of α (1-4) linked glucose with small amounts of α (1-6) branch linkages. Amylopectin is a more complex mixture due to the extensive branching introduced by many more (5%) α (1-6) linkages of the α (1-4) linked chains of glucose. Starch is synthesised by the activity of several enzymes, each of which occur as a number of different isoforms that display tissue specific expression (Fitzgerald, 2004). Amylose content was measured on the F2 rice flour by iodine affinity based colorimetric standard method (Juliano 1971). One hundred (100) mg flour was transferred to volumetric flask and homogenized with 1ml of 95% ethanol and 9 ml of 1N NaOH. The samples were heated for 10 min in the water bath to gelatinize the starch. After cooling, it was made up with 100 ml water. Half ml aliquots of each test solution were separately placed in two test tubes. Five (5.00) ml of water, 0.10 ml of acetic acid and 0.20 ml of iodine solution were added. An additional 4.20 ml of water was added into each tube to make the total volume of reaction mixture to 10 ml. Absorbance was measured using a spectrophotometer (Systronic, India) at a wavelength of 620 nm. The AC was determined using a standard curve developed from known quantities of purified potato amylose from Sigma, USA.

Genotyping of F1lines and F2 seed populatio

DNA isolation of parents and mappin populations

DNA isolation was carried out using standard protocol (Murray and Thomson, 1980). Fresh leaf tissue (21 days old plant) of 1g was taken from FI and parental lines (Tulaipanji and Ranjit) and made pulverised powder with liquid nitrogen in mortar and pestle. The pulverized powdery material was transferred to 15 ml polypropylene tube containing 4 ml of preheated CTAB extraction buffer (2% Cetyltrimethylammonium bromide, 1.5% polyvinyl-pyrrolidone PVP K-30, 1.4M NaCl, 20mM EDTA, 1.7% SDS, 100mM Tris-HCl (pH 8.0), 0.1% β-mercaptoethanol, add before use) with 50µg/ml Proteinase K. Incubated at 65°C in a water bath for 30 minutes with occasional gentle shaking. After cooling at room temperature equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added, and mixed well. Centrifuged at 10,000 rpm for 10 minutes and upper aqueous supernatant was taken out with wide bore Pasture pipette to a new centrifuge tube. DNA was precipitated by adding double volume of chilled (-20ÚC) absolute ethanol followed by addition of 1/ 10th volume of 3M Sodium acetate (pH 5.5) and kept at -20ÚC for overnight for total DNA precipitation.

The precipitated DNA was spooled out and washed 2-3 times in 70% ethanol and dissolved in 500µl RNase A treatment buffer containing 20µg/ml RNase A solution for purification in a 1.5 ml eppendorf tube. It was incubated at 37°C for 1h to remove the RNA contamination. Then extracted with equal volume of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 rpm for 8 minutes and the supernatant was transferred to a new tube. DNA was precipitated by adding double volume of chilled (-20ÚC) absolute ethanol followed by addition of 1/10th volume of 3M Sodium acetate (pH 5.5) and kept at -20ÚC for overnight for total DNA precipitation. The purified DNA pellet was collected by centrifuging at 5000 rpm for 5 minutes in a microfuge. The excess ethanol was air dried and purified DNA was finally dissolved in 250µl TE buffer (pH 8.0) for further use in PCR amplification. Concentration of the purified genomic

DNA in each case was adjusted to10ng/µl in a different aliquots using UV-*vis* Spectrophotometer (Shimadzu-160) and stored at -20°C for further use.

Protocol for PCR amplification

QTL/gene linked six SSR markers was constructed from Operon technology, USA (given below) for genetic diversity analysis of the breeding lines according to McCouch *et al* (2002) and www.gramene.org. PCR amplification was carried out for genotyping the parental/F1 lines and F2 seed population for specific gene(s) or QTLs according to manufacturer protocol (Promega, USA). The 25µl PCR contained 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 200µM dNTPs, 0.2µM primer, GoTaq 1µl (1.25U) and 10µg of genomic DNA as template. PCR amplification was performed in thermal cycler BioRad MJ Gradient (USA) for 35 cycles in the following temperature. 95 f C for 2 min for denaturation and then 35 cycles as follows- 95 f C for 30s, 55 f C for 30s, 72 f C for 30s and one cycle final extension at 72 f C for 7 min and kept at 4 f C forever. The SSR products were resolved in 1.0% agarose gel by electrophoresis at 100 volt for 1h in 0.5X TBE buffer. The gel was stained with ethidium bromide and photographed under UV- transilluminator.

Six primer pairs of SSR markers

Gene and linked SSR marker	**Forward Primers 5′ - 3′**	**Reverse primer 5′ – 3′**
RM 225(AC)	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
Waxy(AC)	CTCTCTCACCATTCCTTCAG	CACAAGCAGAGAAGTGAAGC
sbe2(sbe2)	CCGAGGGAATGCCAGGAGTACCAG	GAACCACAACCAAGTCCAAGGCAA
PTA248 (Xa21)	AGACGCGGAAGGGTGGTTCCCGGA	AGACGCGGTAATCGAAAGGATGAAA
Pita440	CAACAATTTAATCATACACG	ATGACACCCTGCGATGCAA
Pib sub3-5	AGGGAAAAATGCAATGTGC	AGTAACCTTCTGCTGCCCAA

Results and Discussion

F1 seeds were collected from the parent plants after 25 days of pollination and preserved in paper container in a dry place in room temperature (October, 2011). F1 seeds were germinated on pot conMorphological data recording and analysis was carried out according to DUS protocol (Table 1, Fig.1). Plant height during harvesting time was 92 cm on an average of cultivar Tulaipanji, (plant height ranges from 81-95 cm) and mean height was 61.6 cm in case of Ranjit. F1 plant (cross between Tulaipanji and Ranjit) showed 91.2 cm height but 82.8 cm height was measured in case of F1 plant where cross was made between Ranjit and

Table 1. Morphological traits of F1 plants and parental lines

Morphological traits	Tulaipanji	Tulai x Ranjit	Ranjit x Tulai	Ranjit
Plant height (cm)	92 ± 2.24	91.2 ± 1.94	82.8 ± 2.48	61.6 ± 2.94
Stem thickness (cm)	1.38 ± 0.232	1.72 ±0.172	1.66 ±0.307	1.88 ± 0.279
Penaltimate leaf length (cm)	43.34 ± 4.87	43.24 ± 3.39	33.08 ± 1.62	29.46 ± 4.72
Penaltimate leaf breadth	0.86 ± 0.08	0.56 ± 0.08	0.90 ± 0.06	1.02 ± 0.01
Flag leaf length (cm)	21.6 ± 2.31	29.34 ± 3.52	23.34 ± 3.46	21.06 ± 2.49
Flag leaf breadth	0.82 ± 0.04	0.76 ± 0.05	1.04 ± 0.05	1.46 ± 0.10
Panicle length (cm)	24 ± 2.12	18 ± 2.45	18 ± 1.97	21 ± 0.98
Branching	8 ± 1.98	7 ± 3.08	8 ± 2.98	9.5 ± 1.23
Seed/panicle	105± 1.26	80 ± 2.04	96 ± 1.45	123 ± 2.56

Mean of ten reading ± Sd

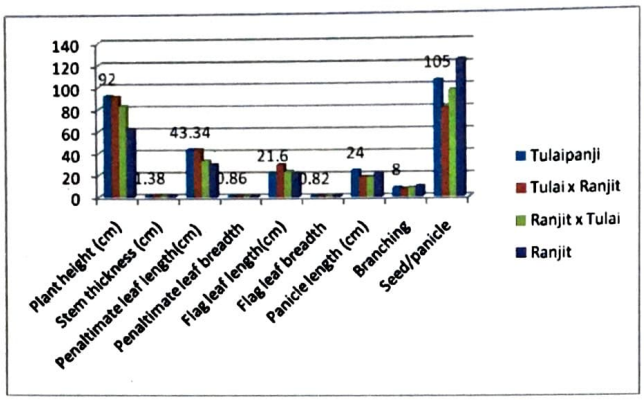

Figure 1. Graphical representation of morphological traits of F1 plants and parental lines.

Tulaipanji. Reciprocal cross showed different results which is not usual. Ranjit can be considered as semi-dwarf plant and Tulaipanji as tall plant as per DUS test. Maturity time is 140-150 days for Tulaipanji and 130-135 days for Ranjit.

F2 grains (seed population) were measured in respect to study their morpho-quality and physicochemical parameters (Table 2, 3 & Fig. 2, 3) - grain length is on an average 7.7 mm with awn (13.5 mm), width 2.1 mm, grain weight 15g/1000 seeds. Kernel length (KL) is measured as 6 mm, considered as medium slender grain according to Govt. of India notification (No. 67, 23 Jan, 2003, Ministry of Commerce), the minimum grain length for A grade basmati rice is 7.0 mm, while its minimum LBR is 3.5, kernel breadth (KB) 1.95 mm and KL/KB ratio is 3.07. Other quality parameters according to DUS protocol are: Aroma-3, ASV- 5, GT-3. Cooked kernel length (CKL) is 8.5 mm, cooked kernel to breadth ratio (CKB) is 2.3 mm, and CKL/CKB ratio is 3.69. The cooked kernel elongation ratio (ELR) is 2.06 in Basmati type grain that elongates length wise with minimal breadth wise swelling on cooking. In contrast, Tulaipanji showed a significantly lower ELR of 1.37 and Ranjit 1.57. Some F2 seed population showed transgressive segregation in respect to GL, GL/GB,

Table 2. Grain quality of F2 seed population based on physicochemical parameters.

Name of the Cultivars	Weight of per seed (mg)	Grain length (mm)	Grain breadth	Ratio of GL/ GB	Awn (mm)	Aroma sensory score	GT	ASV
Tulaipanji	15	7.7 ± 0.02	2.1 ± 0.60	3.6	13.5 ± 0.23	3	3	5
Tulai x Ranjit	17	8.63 ± 0.17	2.5 ± 0.12	3.8	17 ± 0.31	1	3	5
Ranjit x Tulai	17	7.64 ± 0.12	2.5 ± 0.25	3.2	11 ± 0.23	0	1	6
Ranjit	16.29	7.8 ± 0.21	2.5 ± 0.11	3.12	Without awn	0	7	2

Mean of ten reading ± Sd

Table 3. Assessment of genetic variation of F2 seed population based on quality parameters

Name of the Cultivars	Kernel length	Kernel breadth	Ratio of KL/KB	Cooked kernel length	Cooked kernel breadth	Ratio of CKL/ CKB	Linear elongation ratio	Breadth elongation ratio	Cooking time
Tulaipanji	6	1.95	3.07	8.5	2.3	3.69	1.37	1.17	5 min
Tulai x Ranjit	5.8	2.1	2.76	8.0	2.95	2.73	1.36	1.50	5 min
Ranjit x Tulai	5.8	2.1	2.76	8.2	2.96	2.83	1.41	1.50	5 min
Ranjit	5.45	2.15	2.53	7.8	2.3	3.78	1.59	1.06	6 min

Mean of ten reading

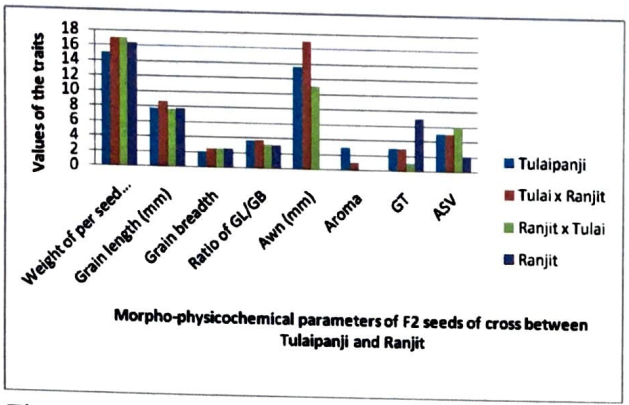

Figure 2. Morpho-physicochemical parameters of F2 seed population.

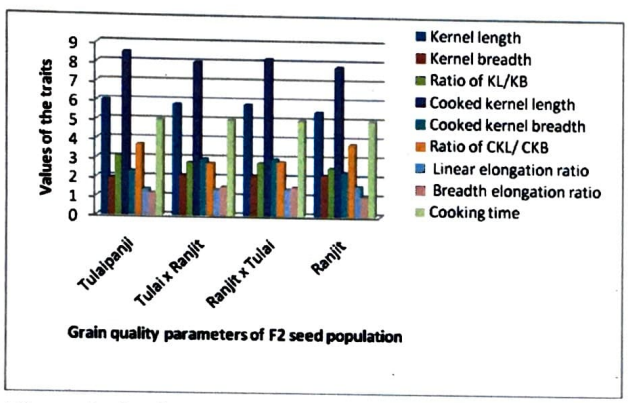

Figure 3. Grain quality analysis of F2 seed population.

awn length because they showed better result in compared to parental lines. Highest aroma was recorded in Tulaipanji at value 3 and F2 grain of cross Tulai x Ranjit showed value at 1. Ranjit as a non scented rice showed aroma value 0 (zero). Ranjit showed highest GT value at 7 but Tulaipanji has low GT value which is 3. Crosses showed GT value ranges from 1-3. Tulaipanji has ASV 5 in compared to 2 in Ranjit. Some F2 grains showed ASV 6. Both the traits are known to be governed by the enzymes of starch biosynthesis pathway, including granule bound starch synthase I (GBSSI- a 60 kDa molecular weight enzyme encoded by the *Waxy* gene locus), soluble starch synthase, and starch branching and de-branching enzymes (Umemoto *et al.*, 2002 & 2004). These enzymes are responsible for the synthesis of amylose during the development of starch granules in cereal endosperm and were not observed in waxy starch. GBSS, which is localized within the starch granule, is responsible for amylose biosynthesis and also has a role in the elongation of long chains in amylopectin. Mutations in the *Waxy* locus leading to loss of GBSSI activity result in amylose free (waxy) starch. While AC is almost entirely attributed to the GBSS1 gene located on the short arm of chromosome 6. The ASV depends on the nature of the amylopectin molecules and is reported to be dependent on soluble starch synthase gene on the same chromosome arm. It could be modulated by the other poorly characterized genes of the pathway. Amylose content of the rice grain determines whether it will be firm and fluffy on cooking, or it will turn sticky and glutinous. The japonica rice varieties have very low AC and hence turn sticky upon cooking, which the consumer prefers in China and Japan for eating with chopstick. Apparent amylose content (AC) is measured 22% in Tulaipanji and 25% in Ranjit. In contrast, basmati type varieties have intermediate AC of 20-25% and their grains remain firm and separated after cooking, at the same time they give a soft mouth feel while eating. Tulaipanji grain showed the same amylase content (22%) and giving the same mouth feel as basmati.

Apparent amylose content (ACC) falls into the following categories: glutinous = 0 to 5%, Low = 5 to 19%, Intermediate = 19 to 23%, and High > 23%. Tulaipanji is intermediate type in respect to amylose contend (22%) and Ranjit is under high category with 25% amylose. Mapping studies to identify quantitative trait loci (QTL) of cooking quality traits showed that amylose, gel consistency and some other viscosity parameters are mainly affected by the *Waxy* gene on chromosome 6 that encodes Granule Bound Starch Synthase I (Tian *et al.*, 2005). None of the studies developed markers for gel consistency, nor determined how the *Waxy* gene actually determines gel consistency. Waxy gene specific QTL was detected in both the parental lines Tulaipanji and Ranjit and also in the F1 breeding lines. Two other SSR markers RM225 and Sbe-2 have also been detected in all the breeding lines and associated with amylose biosynthesis QTLs (Fig. 5). Most of these grain quality attributes are controlled by quantitative trait loci (QTLs) as inferred from continuous phenotypic variation in the segregating progeny of intervarietal crosses. Genetics of rice quality has also been studied in various genetic backgrounds using molecular

Figure 5. PCR amplified SSR marker (Waxy and Sbe2 marker) specific bands fractionated on 1% agarose gel. Lane 1-for Waxy gene and lane 2-4 for Sbe2 gene in rice cultivar Tulaipanji.

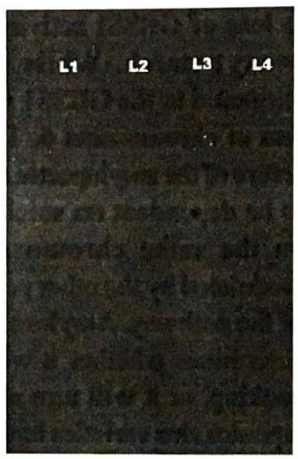

Figure 6. SSR marker (for Xa21 and Pita440) specific PCR amplified products were separated on 1% agarose gel. Marker Xa21 in rice cultivar, lane 1- Tulaipanji, no amplification, lane 2- F1 plant Tulai x Ranjit, showing band, lane 3-4, no marker specific band for Pita440 in Ranjit and Tulaipanji.

markers (Wang *et al.*, 1995). The F2 seed showed transgressive segregation for the traits GL, GL/GB ratio, and awn length (Table 2).

QTL for amylose content

QTL for amylase content was detected on short arm of chromosome 6 linked with SSR marker such as Waxy, Sbe-2 and RM225. The Waxy QTL was located in the waxy gene (GBSS1) region of chromosome 6.

QTLs for leaf blight and blast

Amplified PCR band was observed while Xa21 specific primer was mixed in the reaction of parental and breeding lines. Xa21 specific band was present in parental plant Ranjit (Fig. 6) and in cross between Tulaipanji and Ranjit but not in Ranjit x Tulai. Ranjit can be used as donor plant for bacterial blight resistance gene Xa21. Disease resistance QTL/ genes for bacterial blight has been identified (Xa genes) and introgressed into HYV (Xa21, Song *et al.*, 1995) from wild rice (*Oryza longistaminata*) and introgressed successfully into the cultivated rice to protect against the biotic stress and located on chromosome 11. Bacterial leaf blight (BLB) is caused by *Xanthomonas oryzae* pv. *oryzae* (that is why marker name is Xa). Blast resistance gene (RM529, Pib-sub 3-5, and Pita440) specific band have not been amplified in any one of the parental or breeding lines, while gene specific primer was used (Fig.6). Which suggested that blast resistance gene (QTLs) was not exist in any one of the parental lines. Blast disease of rice caused by the filamentous ascomycete *Magnaporthe oryzae* (*Magnaporthe grisea*) or *Pyricularia oryzae* (teleomorph, *Magnaporthe oryzae*) is a continuous thread to rice production and global food supply. Blast is recorded one of the main diseases of rice because of its worldwide distribution and destructiveness under favourable conditions. Rice blast has been reported in nearly every rice production region in the world and great concern in rice yield (Khush and Jena, 2009). R-gene Pita, a single copy gene, is located at 10.6 Mb near the centromere of chromosome 12, a region that often associates with recombination suppression (Chen *et al.* 2002). Resistance to *M. oryzae* in rice follows a gene-for-gene specificity where major resistance R genes (***Pi*** for ***Pyricularia***) are effective in controlling infection by races of *M. oryzae* possessing corresponding avirulance (AVR) genes (Flor 1971; Jia *et al.* 2000). Until now, at least 40 major ***Pi*** genes have been cloned (McCouch *et al* 1998, Chen *et al.* 2006; Liu *et al.* 2007). Out of these only Pita440 and Pib-sub 3-5 were used in the PCR reaction to study their existence in the parental lines. But no parental lines showed its presence in the PCR amplified band.

Grain and cooking quality traits are economically important for the traders and consumers of Tulaipanji rice which is comparable to Basmati rice to some extent. Therefore, new high-yielding disease tolerant variety of Tulaipanji rice need to be developed to cater for the growing domestic demand for this premium grade non-basmati aromatic rice of North Dinajpur district. Molecular markers tightly linked to the major QTLs governed the quality traits can be used in marker assisted breeding to develop new Tulaipanji variety to maintain its unique quality attributes while

introgressing the yield potential QTLs and resistance QTLs associated to various biotic (Xa21, Pita440, Pib-sub 3-5) and abiotic stresses.

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Antioxidative responses of mandarin plants to water stress

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Abstract
Citrus reticulata grows in warm climatic conditions. Stresses such as water logging, drought, soil acidity, unbalanced nutrition and pathogenic infestation lead to root injury resulting in citrus tree decline and therefore huge economic losses. In order to determine the effect of flooding and drought on mandarin plants, mandarin plants were subjected to water logging condition in the field and in pots for drought condition. Biochemical and morphological changes induced by water logging and drought conditions were determined. The plants showed slight wilting and leaf dropping by the third day of flooding whereas in drought the leaves curled up, became crisp and later dried out. Marked changes in antioxidative enzymes such as peroxidase, catalase and ascorbate peroxidase was observed during stress in comparison to control plants. Antioxidative activity was seen to be more in the leaves than in the roots. Among the antioxidants carotenoid content showed a significant decrease during the flood stress but increased in drought stress. An increase in ascorbate content was observed during stress in comparison to the control. From this study, we can conclude that water stress causes adjustment of antioxidant balance in mandarin plants.

Keywords: Mandarin, drought, flood

Water stress in citrus reduces stomatal conductance, transpiration rate and net assimilation of carbon dioxide (Arbona *et al.*, 2005; Garcia-Sanchez *et al.*, 2007). Waterlogging has a profound effect on root metabolism, mainly through the deficiency of oxygen (Drew 1997). In tropical and subtropical regions, severe crop losses are caused by prolonged seasonal rainfall. Previous work has shown that there is potential for water damage to citrus trees if roots are submerged in water for four days or more during frequent extended summer rains. During the cooler months of December through February, citrus trees can tolerate flooded conditions for longer periods than during the hot summer months. (Boman and Tucker, 2002). Sour odour in roots indicate an oxygen deficient environment. The presence of hydrogen sulfide (a rotten egg odour) is an indication that fresh feeder roots are dying. Anaerobic bacteria (which grow only in the absence of oxygen) develop rapidly in flooded soils and contribute to the destruction of citrus roots. Root damage symptoms include leaf yellowing, chlorosis, wilting, fruit drop, leaf drop and dieback. Excess water produces anoxic soil conditions within a few hours (Gambrell and Patrick, 1978). Flooding stress is usually less when water is moving than when water is stagnant for anaerobics cannot multiply if oxygen is present.

Flooding will also affect many other biochemical and physical processes taking place in the rhizosphere (He *et al.*, 1996). Flooding of the root system arrests growth and diminishes the productivity in the majority of terrestrial plants (Limkemer *et al.*, 1998).

Drought stress is considered to be a moderate loss of water, which leads to stomatal closure and limitation of gas exchange. Desiccation is much more extensive loss of water, which can potentially lead to gross disruption of metabolism and cell structure and eventually to the cessation of enzyme catalyzed reactions (Smirnoff 1993; Jaleel *et al.*, 2007).

Drought limits canopy development and inhibits vegetative and fruit growth. In many plant species, root hydraulic conductance decreases significantly under water deficit situations (Sumner & Boswell, 1981; Cruz *et al.*, 1992; North and Nobel, 1996; North *et al.*, 2004). Plants have also developed various mechanisms such as higher root-shoot ratios, fewer and smaller leaves, concentrated solutes or increased activity of oxidative stress

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enzymes in leaf cells to withstand water stress (Lei *et al.*, 2006).

Drought stress is characterized by reduction of water content, diminished leaf water potential and turgor loss, closure of stomata and decrease in cell enlargement and growth .Severe water stress may result in the arrest of photosynthesis, disturbance of metabolism and finally the death of plant (Jaleel *et al.*, 2008)

Drought is one of the most common environmental limitations affecting growth and productivity of plants, and causes many metabolic and oxidative changes in plants (Reddy *et al..*, 2004) and recent global climate change has made this situation more serious (Pan *et al.*, 2002)

Considering the importance of water stress in mandarin the present work was undertaken to determine the effects of flooding and drought stress in mandarin plants.

Materials and Method

Plant material

Four year old mandarin (*Citrus reticulata*) plants were obtained from Kalimpong, Mirik, Bijanbari and Teesta Valley orchards. These were then maintained in the experimental plot of the Department. One set of plants were maintained in the field whereas another set was planted in pots.

Water stress treatment

For flooding, the plants in the field were flooded with water and flooding condition was maintained for desired period. In case of drought, potted plants were left without water for the desired period.

Extraction and assay of enzyme activities

Preparation of enzyme extract

The leaves and roots collected from treated and control plants were ground to fine powder with a mortar and pestle under liquid nitrogen in cold using respective buffers containing 1% (w/v) polyvinylpolypyrrolidone. The homogenate was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was directly used as crude extract for enzyme assays.

Assay of activities

Peroxidase (POX: EC. 1.11.17)

Peroxidase activity was assayed following the method described by Chakraborty *et al.* (1993) spectrophotometrically in UV VIS spectrophotometer (Model 118 SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity was expressed as "A_{460} mg protein^{-1} min^{-1}.

Ascorbate peroxidase (APOX : EC.1.11.1.11)

Activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada and Takahashi (1987) with some modification. Enzyme activity was expressed as ΔA_{290} mg protein^{-1} min^{-1}.

Catalase (CAT: EC.1.11.1.6)

Catalase activity was assayed as described by Beers and Sizer (1952) by estimating the breakdown of H_2O_2 which was measured at 240 nm in a spectrophotometer. The enzyme activity was expressed as ΔA_{245} mg protein^{-1} min^{-1}.

Phenylalanine Ammonia Lyase (PAL: EC.4.3.1.5)

Phenylalanine Ammonia Lyase was extracted and estimated following the method described by Chakraborty *et al.* (1993).

Proline

Free proline was extracted and estimated following the method of Bates *et al.* (1973). Fresh leaf material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman's No. 2 filter paper. The reaction mixture was extracted with 4 ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and absorbance was measured at 520 nm.

Protein

Protein contents in each case were extracted from leaves and roots of maize by following the method of Chakraborty *et al.* (1995) and estimated by following the method of Lowry *et al.* (1951).

Ascorbate

Ascorbate was extracted and estimated by

following the method of Mukherjee and Choudhuri (1983). The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

Carotenoids

Carotenoids were extracted and estimated following the method described by Lichtenthaler (1987). Extraction was done in methanol and the extract was filtered. Absorbance of the filtrate was noted at 480nm, 663nm and 645nm in a VIS spectrophotometer and the carotenoid content was calculated using the standard formula.

Results and Discussion

In the present study, citrus seedlings obtained from the four locations- Kalimpong (KL), Bijanbari (BJ), Mirik (MR) and Teesta Valley (TV) showed varying responses to flooding and drought (Fig. 1A & Fig. 1B). Among the four, the plants obtained from Bijanbari seemed to be more susceptible. Like other crops, citrus trees respond to flooding by reducing leaf water potential, stomatal conductance, gas exchange and plant growth (Vu and Yelenosky, 1991). During prolonged periods of soil flooding, a decrease in root hydraulic conductance (Ruizsanchez *et al.*, 1996; Syvertsen *et al.*, 1983) causes impairment of water uptake, which eventually leads to leaf wilting and chlorosis (Arbona *et al.*, 2008).

The antioxidant defense machinery protects plants against oxidative stress damages. Plants possess very efficient enzymatic (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase, guaicol peroxidase, and glutathione-S-transferase) antioxidant defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS (Gill and Tuteja, 2010).

Catalase (Fig.2A and B) and ascorbate peroxidase (Fig. 2C and D) activity decreased in almost all the samples during both types of stresses but peroxidase (Fig. 3A and B) activity mostly increased in leaf specially during both drought and

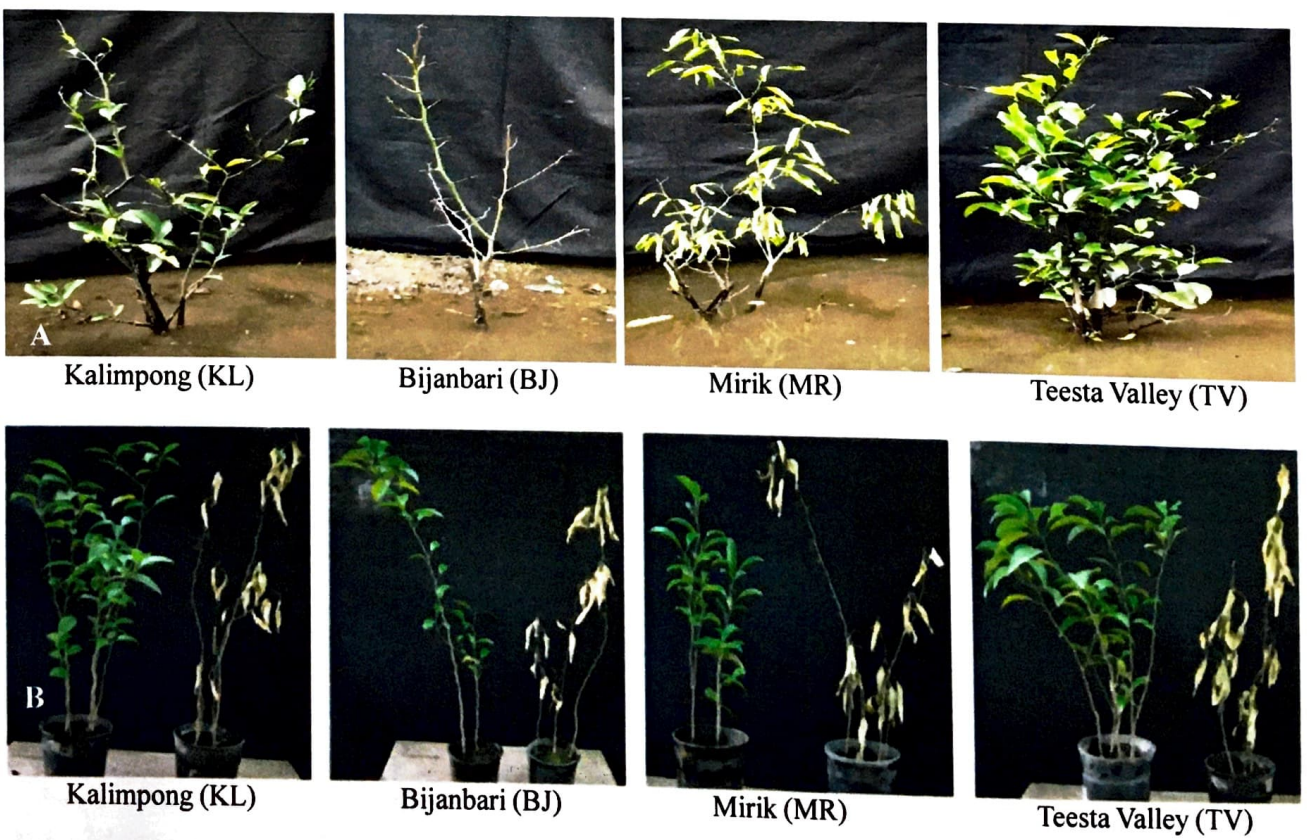

Fig. 1: Responses of mandarin plants from different locations to flood (A) and drought (B)

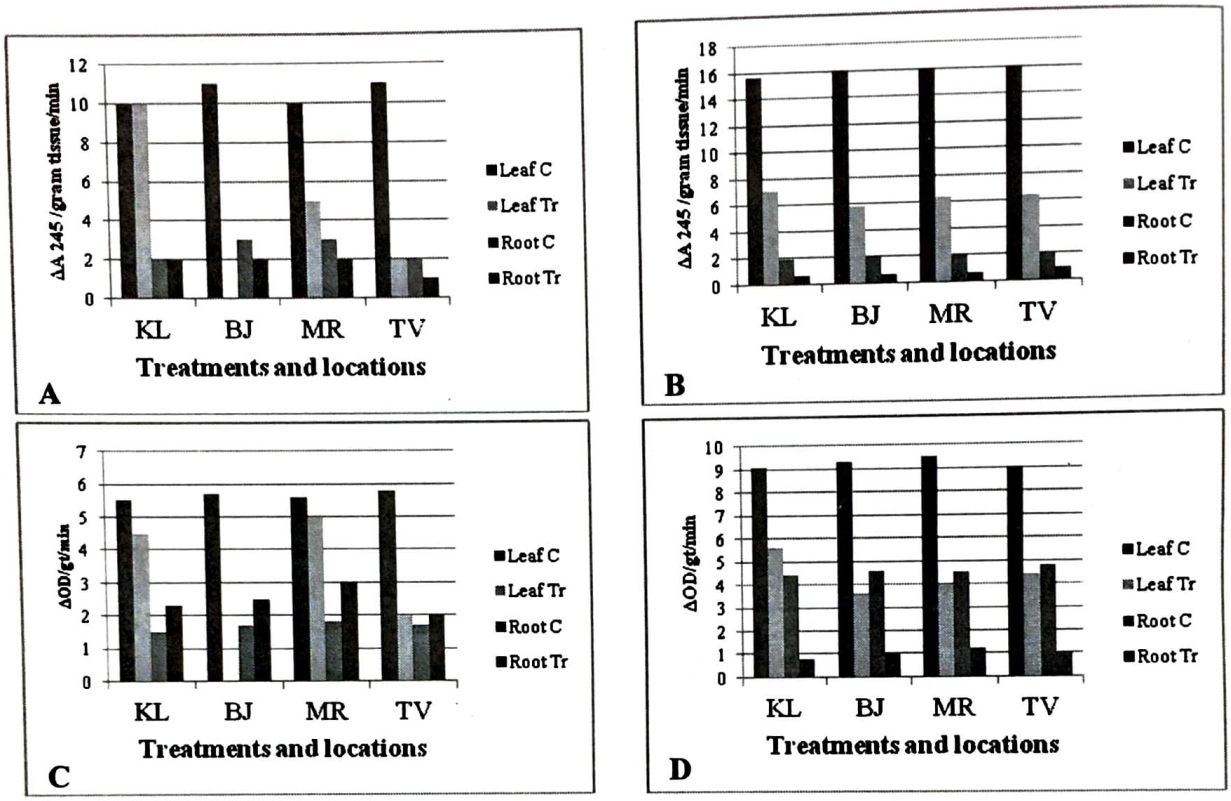

Fig. 2: Catalase (A&B) & Ascorbate peroxidase (C&D) activities during flooding (A&C) and drought (B&D)

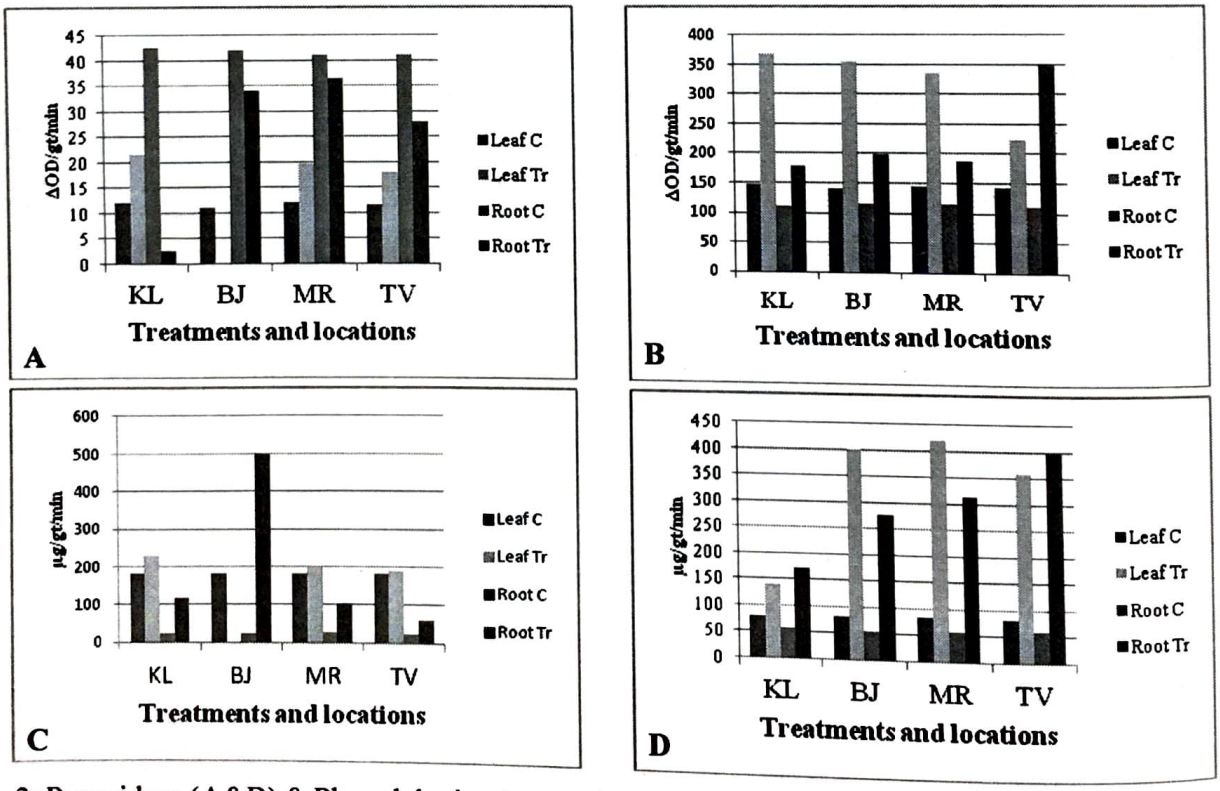

Fig. 3: Peroxidase (A&B) & Phenylalanine Ammonia Lyase (C&D) activities during flooding (A&C) and drought (B&D)

flooding stress indicating that the peroxidase activity plays a vital role in scavenging of free radicals during water stress in mandarin plant. It has been reported that catalase activity declined during stress in Dhanya and Swarna varieties of maize while the other two varieties showed an increase during dought stress (Lama and Chakraborty, 2012). Maintaining a relative higher antioxidants activity may lead to drought tolerance by improving the capacity to cope with ROS (Sharma and Dubey, 2005).

PAL activity showed varied trends in samples subjected to flooding stress, the activity increased in some samples during stress in both leaf and root whereas in some samples the activity decreased with flooding stress (Fig. 3C). But in case of drought (Fig. 3D) the activity increased in both the leaf and the root of the samples in comparison to the control plants. Maximum activity was observed in BJ, MR and TV in both the leaf and roots of these samples.

Ascorbate content showed an increase in both flooding and drought stress in all the samples of mandarian (Table 1) but carotenoid content showed a different trend in two type of water stress where a decrease in the carotenoid content was observed in flooding (Table 1) whereas there was an increase in carotenoid content during drought. Accumulation of ascorbate and carotenoids was enhanced

Table 1: Ascorbate and carotenoid content during flooding and drought

Plant	Ascorbate (µg/g tissue)				Carotenoid (mg/g tissue)			
	Drought		Flood		Drought		Flood	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
KL	4	6.50	9.26	10.78	0.006	0.025	0.027	0.026
BJ	4.11	7.75	9.23	0.000	0.005	0.010	0.027	0.000
MR	4.15	7.64	9.11	18.70	0.002	0.017	0.027	0.016
TV	4.19	7.30	9.12	14.32	0.004	0.018	0.027	0.020

significantly following water stress in both the varieties, but it was greater in variety 30P30 (Chakraborty and Lama, 2010).

Protein content in all the samples was more in the leaves than in the root during both drought and flooding stress (Table 2). Under anoxia conditions, the pattern of protein synthesis is altered in plants. The proteins which are synthesized as a specific response to anaerobes are called anaerobic polypeptides (ANPs) (Sachs *et al.*, 1980).

During drought stress proline (Table 3) content increased in all the four samples of mandarin plant. Both leaves and root showed an increase during drought stress but there was more increase in the roots than in the shoots. Proline which is known to occur widely in higher plants, normally accumulateds in large quantities in response to environmental stresses (Kavikishore *et al.*, 2005; Ashraf and Foolad, 2007). Accumulation of osmolytes such as proline, helps in maintaining cell water status, sub-cellular structures and protecting membranes and proteins from the denaturing effects of the osmotic stress (Ashraf and Foolad,

Table 2: Protein content of leaf and root during flooding and drought

Plant	Protein (Leaf) (mg/g tissue)				Protein (Root) (mg/g tissue)			
	Drought		Flood		Drought		Flood	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
KL	150.5	192.0	235	182.5	15.0	26.0	55.0	70.0
BJ	150.5	200.0	235	0.000	15.0	24.0	55.0	75.0
MR	150.5	196.0	235	225.6	15.0	24.0	55.0	62.5
TV	150.5	196.0	235	200.0	15.0	26.0	55.0	50.0

Table 3: Proline content of leaf and root during drought

Plant	Proline (Leaf) (mg/g tissue)		Proline (Root) (mg/g tissue)	
	Control	Treated	Control	Treated
KL	0.56	0.59	0.9	1.34
BJ	0.5	1.23	0.96	1.76
MR	0.52	1.94	0.96	2.12
TV	0.51	1.52	0.94	3.36

2007).With the increase in intensity of drought there was an increase in both proline and ascorbate content in all varieties of maize (Lama and Chakraborty, 2012).

PAGE analyses was performed in plant sample subjected to drought stress and new bands of isoform was observed in leaf and root of plant sample subjected to drought stress (Fig. 4). Being a self-regulating system, the plant is capable of developing a survival strategy under stressful conditions. It should be borne in mind that the action of stress factor on the underground plant part causes a series of physiological changes in the aboveground organs; these changes reflect either plant adaptation or damage (Krishtetter *et al.*, 1998). As a result of the above study we can conclude that mandarin plants do have the ability to cope with both kinds of stress to some extent.

It is known that plant adaptation to stress conditions requires additional material and energy resources,

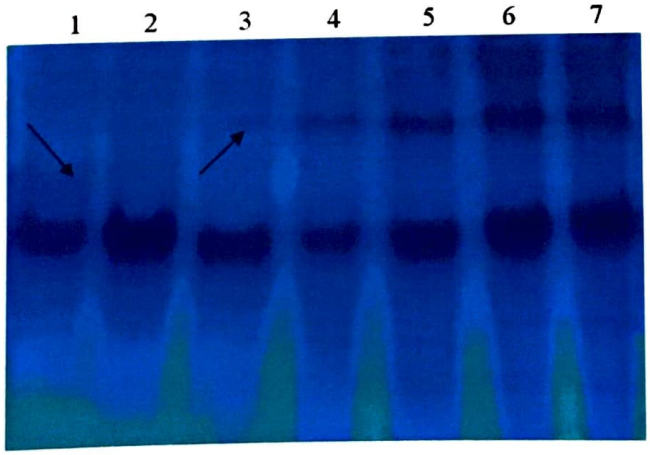

Fig 4: Isozyme analysis of peroxidase from leaves and roots of mandarin. 1- Control leaf; 2 & 3- Drought treated leaves - KL & BJ, respectively; 4 Control root; 5-7 Drought treated roots - KL, BJ & MR respectively.

making processes such as photosynthesis and respiration more significant (Semikhatova 2000). Soil water logging and submergence (collectively termed flooding) and drought are abiotic stresses that influence species composition and productivity in numerous plant communities, world-wide. Hydrological patterns can determine the vegetation in natural and man-made wetlands, since this is dependent on ecophysiological responses of species to flooding (e.g. Voesenek *et al.*, 2004). For most other crops, excess water is a major constraint which adversely affects grain yields (Setter and Waters, 2003) and growth of pasture species (Gibberd and Cocks, 1997; Gibberd *et al.*, 2001). Drought triggers a wide variety of plant responses, ranging from cellular metabolism to changes in growth rates and crop yields. Understanding the biochemical and molecular responses to drought is essential for a holistic perception of plant resistance mechanisms to water-limited conditions (Anjum *et al.*, 2011).

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Present status of flora, fauna and vegetation structure in the wetlands of Maldah district of West Bengal, India

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Abstract
The highest area of fresh water wetlands in the West Bengal is situated in the district of Maldah. Out of 23 larger wetlands of West Bengal 11 are located in this district. Maldah-wetlands are quite old and natural with good biodiversity and healthy ecosystem. These wetlands are the house of around 351 vascular plant species and several animal species like waterfowl, fishes, reptiles, amphibians, mollusks and numerous insects. The local people largely depend also on these wetlands for their sustenance. But several threats, mainly anthropogenic, are gradually destroying these important ecosystems since the last decade.

Key words: Wetland, Maldah, Biodiversity, Flora, Socio-economy, Threats.

Introduction:

Innumerable and wide range of wetlands are distributed throughout West Bengal including fresh water lakes of Darjeeling hills to the marine wetlands of Sundarbans of South 24 Parganas district. The fresh water marshes of Maldah fall under the sub-Himalayan wetland system of the state. Wetland areas are also called ecotonal zones where more than two habitats meet. Wetlands support both the aquatic and the terrestrial life forms. According to Tiner (1999) 'wetland' is a generic term used to define universe of wet habitats including marshes, swamps, bogs, fens and similar areas. Maldah district is covered with second highest wetlands areas in West Bengal just after 24 Parganas (North & South). The water bodies or wetlands in this area are generally lies along with different major river systems. In nature, the Maldah wetlands are perennial and seasonally flooded fresh water bodies. Most of those are very old and supports rich biological elements. These wetlands are of mainly *Palustrine* (annual or perennial marshy land), *Riverine* (oxbow lake or small streams) and *Lacustrine* (lakes & ponds) types. While first two types are very old and naturally originated, lakes and ponds are mostly artificially created few centuries back by different kings and *nawabs* ruling over different parts of this region. Considering fresh water wetlands the district of Maldah holds first position based on the number as well as total area of wetlands in this state. According to Meenbarta (1998) highest number of wetlands or water bodies in West Bengal is present in this district. Data presented in Census report provided by Ministry of Environment & Forests, Govt. of India recorded 11 large fresh water wetlands, out of 23 in West Bengal, present in different blocks of this district (Sharma, 2003). Maldah district is located between the 24° 40′ 20″ and 25° 32′ 08″ N latitude and between 87° 45′ 50′ ′ and 88° 28′ 10″ E longitude with an average altitude of 61 m over mean sea level. Out of the total 3733 sq km area of the district, 156.76 sq km area is occupied by water bodies of less than 10 hectors size and 273.89 sq km areas is covered by wetlands of over 10 hectors in size (Sharma, 2003). Other wetland types like mudflat, sandbanks, marshy lands and low lying areas covers 450.38 sq km, 78.52 sq km, 120.34 sq km and 47.27 sq km respectively, for which the total comes to 645 sq km (Raha *et al*, 1994) of wetland. Recurring floods, almost every year, gradually increases the wetland areas in the district.

A total of 1076 sq km land i.e. almost 30% area of the district is occupied by wetlands. During

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monsoon most of the wetlands merg altogether to forms two-three larger water bodies and covers almost around 60% of the total land area of the district. These large water bodies are directly or indirectly connected with different rivers like *Ganga, Fulhar, Pagla, Mahananda, Tangan, Punarbhaba, Chitola, Kalindri* etc.

Recent statistics, based on satellite data, estimated by the Institute of Environmental Studies & Wetland Management, Kolkata (IESWM) that total wetland area of Maldah is 29416.95 hectors, which is 7.88% of its total geographical area (Bhattacharyya *et al,* 2000). This area reduces to 6844.53 hectors during pre-monsoon and covers 15191.58 hectors in post-monsoon seasons. Out of the total wetland area 28750.68 hectors i.e. 97.73% of wetland is natural, whereas 665.78 hectors i.e. 2.23% is manmade. Detail informations on Maldah wetlands are provided in Table 1. The largest number of 235 seasonal waterlogged natural wetlands represent the most abundant type that contributes 20956.49 hectors area of wetland for the district (Bhattacharyya *et al,* 2000).

Table 1: Distribution of wetland types and areas in Maldah district (*Source*: Bhattacharya *et al* 2000)

Nature of Wetland		Classes of Wetland	Number	Area (ha)
INLAND	Natural	Pond	22	2760.79
		Cut off Meanders/Oxbow Lake	190	2986.80
		Marsh/Swamp	20	2047.09
		Waterlogged seasonal	235	20956.49
	Manmade	Reservoir	4	34.86
		Tanks	90	613.07
		Waterlogged	1	17.85
		Ash pond	-	-
		Total	562	29416.95

Methodology:

Several vegetational surveys had been undertaken in the study area in different seasons since 2003. Plant specimens were collected by random sampling. To record plants in their different phenophases and the time of appearance in the different depths of water in different seasons, same area has been visited repeatedly round the year. Plant specimens were processed into dried, poisoned and mounted herbarium sheets following Jain & Rao (1977). The voucher specimens were identified and preserved in the NBU-Herbarium. Plants were identified using available Floras (Hooker, 1872-1897; Prain, 1903; Deb, 1957; Hara, 1966, 1971; Guha Bakshi, 1984; Grierson & Long, 1987-1999; Cook, 1996) and finally matching at CAL and BSHC. In addition, many interesting birds, fishes and other animals were also photographed. The random interview of local people also helped to collect and/or understand different socio-economic relations and imposing threats faced by the flora and the vegetation.

Observation & Discussion:

The wetlands are locally called as *Beel* or *Beeloth, Dighi, Khal* etc. Most of the wetlands were naturally originated due to sifting of different rivers – oxbow lakes. Apart from natural wetlands there are some artificial or manmade water bodies of which few are hundreds of years old. The nature of a wetland generally depends mostly on the topography of land. The district is composed of three dominating topography namely *Diara, Barind* and *Tal* (Fig 1). The wetlands of Maldah can be divided into the following types based on their origin:

1. Marshes and floodplains of *Barind* region along with small lakes and ponds
2. Marshes and seasonally flooded wetlands of *Tal* region, and

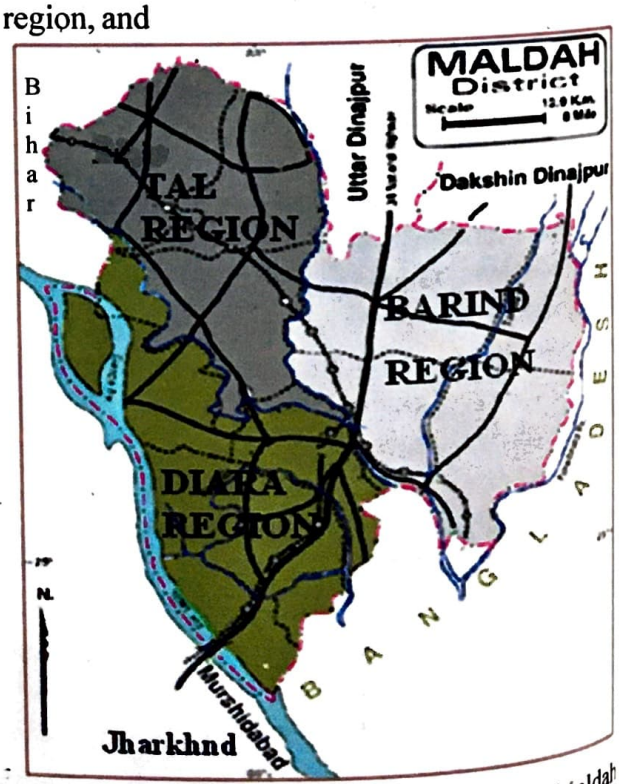

Fig. 1. Wetland Types and their areas in the Maldah District of West Bengal

3. Marshes and seasonally waterlogged swamp wetlands of *Diara* region along with few lakes.

The wetland areas lie in between terrestrial and aquatic ecosystems, so these are characterized by two adjacent ecosystems i.e. aquatic, marsh and terrestrial habitat. Such wetlands are also considered as *ecotonal* habitat (Mitsch & Gosselink, 1993) that clearly shows their capacity to support rich biodiversity suitable for aquatic, marsh and upland vegetation types.

The wetlands of Maldah district are very old and majority of those are naturally originated and developed the ecosystem through suitable seral stages since their origin. The yearly water cycle makes the wetlands more healthy and productive. A small patch of natural vegetation of *Barringtonia acutangula* (Linnaeus) Gaertner (Lecythidaceae) of *Tilasan* areas is a swamp forest situated at the Basin of Punarbhaba River across the Indo-Bangladesh International Boarder. This is the only natural patch of *Barringtonia* vegetation and is a part of the Nayabandh beel complex that includes *Chakla, Bakla, Ramdole* and *Tilasan beels* within the boundary of West Bengal. The wild biodiversity of Maldah district is mostly restricted in and around the wetlands areas, but are suffering from acute stresses, mainly anthropological and some natural.

The wetland and aquatic flora of Indian sub-continent was studies by several authors including Duthie (1903-1929); Biswas & Calder (1935, 1955); Subramanyam (1961); Deb (1962); Jha (1965); Naskar (1990); Gopal (1995); Ghosh (1994) and Cook (1996). Present study enlisted 345, species, representing 200 genera of flowering plants are growing wild in these water bodies. Out of these 198 species are dicots and the rest are monocots. Six species of 6 different genera of pteridophytes and 4 species of bryophytes are also recorded. The dominating 15 families in such vegetation are presented in Table 2.

Table 2: Fifteen most dominating families

Sl. No.	Family	Genus	Species	%
1	Poaceae	34	52	15.07
2	Cyperaceae	8	47	13.62
3	Asteraceae	24	29	8.41
4	Scrophulariaceae	9	22	6.38
5	Euphorbiaceae	6	10	2.90
6	Polygonaceae	2	11	3.19
7	Amaranthaceae	4	9	2.61
8	Lythraceae	3	9	2.61
9	Acanthaceae	4	8	2.32
10	Fabaceae	4	8	2.32
11	Araceae	5	7	2.03
12	Commelinaceae	3	7	2.03
13	Convolvulaceae	4	6	1.74
14	Hydrocharitaceae	5	5	1.45
15	Solanaceae	4	5	1.45

The floral composition of different wetland vegetation can be categorized under following sub-groups:

Algal component:

Different types of algae are the common components in the wetland vegetation of Maldah. They are either free living or remain attached to different types of substrata. Common algae found to grow includes different species of *Oedogonium, Lyngbea, Nostoc, Ulothrix, Oscillatoria, Spirogryra, Volvox, Hydrodyctyon, Voucharia, Chara, Nitella* etc.

True aquatic Plants:

The true aquatic plants are growing strictly in water and can be grouped as (i) *Free floating* [e.g. *Eichhornia crassipes* (Martius) Solms, *Lemna perpusilla* Torrey, *Spirodella polyrhiza* (Linnaeus) Schleid etc.]; (ii) *Submerged plants* [*Vallisneria spiralis* Linnaeus, *Nechamendra alternifolia* Thwaites, *Najas graminea* Delile, *Potamogeton crispus* Linnaeus, *Aponogeton natans* (Linnaeus) Engler & Krause, *Aponogeton crispus* Thunberg etc.]; (iii) *Rooted with floating leaved* [*Nymphaea nouchali* Burman f., *Nymphaea pubescens* Willdenow, *Nelumbo nucifera* Gaertner, *Nymphoides indica* (Linnaeus) Kuntze, *Nymphoides hydrophylla* (Loureiro) Kuntze etc.]; (iv) *Suspended* [*Ceratophyllum demersum* Linnaeus, *Utricularia aurea* Loureiro, *Utricularia gibbosa* Linnaeus ssp. *exoleta* (R. Brown) P. Taylor, *Utricularia inflexa Forsskal* ssp. *stellaris* (Linnaeus f.) Taylor etc.]; and

(v) *Amphibians* [*Sagittaria guayanensis* Humboldt, Bonpland & Kunth, *Sagittaria sagittifolia* Linnaeus, *Limnophila heterophylla* (Roxburgh) Bentham, *Limnophila repens* (Bentham) Bentham, *Limnophila indica* (Linnaeus) Druce, *Limnophila sessiliflora* (Vahl) Blume, *Ammannia baccifera* Linnaeus, *Ammannia multiflora* Roxburgh, *Rotala rotundifolia* (Buchanan-Hamilton) Koehne, *Bergia ammannioides* Roxburgh, *Monochoria vaginalis* (Burman f.) K. Presl, *Monochoria hastata* (Linnaeus) Solms, *Monochoria vaginalis* (Burman f.) C. Presl *ex* Kunth etc.].

Semi-aquatic Plants (Helophytes):

Among the plants those are recorded from this area most of the plants are semi aquatic (220 species) and most dominating species are *Rungia pectinata* (Linnaeus) Nees, *Alternanthera paronychioides* St. Hilaire, *Alternanthera sessilis* (Linnaeus) R. Brown *ex* DC., *Amaranthus viridis* Linnaeus, *Amaranthus lividus* Linnaeus, *Celosia argentea* Linnaeus, *Digera muricata* (Linnaeus) Martius, *Vetiveria zizanioides* (Linnaeus) Nash, *Centipeda minima* (Linnaeus) A. Brown & Ascherson, *Gnephalium luteo-album* ssp *affine* (D. Don) Koster, *Grangea maderaspatana* (Linnaeus) Poiret, *Thespis divaricata* DC., *Oenanthe javanica* Linnaeus, *Kyllinga brevifolia* Rottboell, *Fimbristylis littoralis* Gaudich, *Fimbristylis dichotoma* (Linnaeus) Vahl, *Bulbostylis densa* (Wallich) Handle-Mazzetti *ex* Karsten & Schenck, *Cyperus compactus* Retzius, *Desmostachya bipinnata* (Linnaeus) Stapf , *Leptochloa panicea* (Retzius) Ohwi, *Echinochloa colona* (Linnaeus) Link, *Echinochloa crus-galli* (Linnaeus) P. Beauverd, *Eragrostis unioloides* (Retzius) Nees *ex* Steudel, *Mariscus compactus* (Retzius) Boldingh, *Cyperus digitatus* Roxburgh, *Cyperus difformis Linnaeus, Cyperus iria* Linnaeus, *Imperata cylindrica* (Linnaeus) Raeuschel, *Leersia hexandra* Swartz, *Sacciolepis interrupta* (Willdenow) Stapf, *Saccharum spontaneum* Linnaeus, *Eleocharis palustris* R. Brown, *Eleocharis tetraquetra* Nees, *Eleocharis atropurpurea* (Retzius) Kunth, *Schoenoplectus articulatus* (Linnaeus) Palla, *Schoenoplectus juncoides* (Roxburgh) Palla, *Schoenoplectus lateriflorus* (Gemlin) Lye, etc.

Pseudo-wetland Plants:

Except few perennial water bodies most of the wetlands of Maldah district are seasonally waterlogged. During summer these areas are completely dried out and some species of upland plants start growing there. The dominating upland plants of these wetlands including *Amaranthus spinosus* Linnaeus, *Ageratum conyzoides* Linnaeus, *Blumea lacera* (Burman *f.*) DC., *Cirsium arvense* (Linnaeus) Scopoli, *Elephantopus scaber* Linnaeus, *Eupatorium odoratum* Linnaeus, *Mikania micrantha* Kunth, *Parthenium hysterophorus* Linnaeus, *Vernonia cinerea* (Linnaeus) Lessing, *Cyanoglossum lanceolatum* Forsskal, *Operculina turpethum* (Linnaeus) S. Manso, *Chrozophora rottleri* (Geiseler) Jussieu *ex* Sprengel, *Croton bonplandianus* Baillon, *Alysicarpus monilifer* (Linnaeus) DC., *Mimosa pudica* Linnaeus, *Senna sophera* (Linnaeus) Roxburgh, *Piper Longum* Linnaeus and *Solanum sisymbriifolium* Lamarck.

Plants of lowland crop fields:

Sallow-watered areas of wetlands are generally used for paddy and jute cultivation where submerged or dried floor are dominating with several weeds like *Digera muricata* (Linnaeus) Martius, *Caesulia axillaris* Roxburgh, *Cortula anthemoides* Linnaeus, *Eclipta prostrata* (Linnaeus) Linnaeus, *Enydra fluctuans* Loureiro, *Ixeris polycephala* Cassini, *Sonchus asper* (Linnaeus) Hill, *Centella asiatica* (Linnaeus) Urban, *Ipomoea aquatica* Forsskal, *Stellaria wallichiana* Bentham *ex* Haines, *Lobelia alsinoides* Lamarck, *Bergia ammannioides* Roxburgh, *Fumaria indica* (Haussknecht) Pugsley, *Hydrolea zeylanica* (Linnaeus) Vahl, *Hypericum japonicum* Thunberg *ex* Murray, *Veronica anagallis-aquatica* Linnaeus, *Microcarpaea minima* (J. Koenig *ex* Retzius) Merrill, *Lindernia parviflora* (Roxburgh) Haines, *Phyllanthus amarus* Schumacher & Thonning, *Ammannia baccifera* Linnaeus.

Apart from above categories several interesting plants are also recorded. These include insectivorous plants like *Utricularia aurea*

Loureiro, *Utricularia gibbosa* Linnaeus ssp. *exoleta* (R. Brown) P. Taylor, and *Utricularia inflexa* Forsskal ssp. *stellaris* (Linnaeus *f.*) Taylor, which are growing there in wild. Some species like *Oryza rufipogon* Griffith, *Oryza nivara* Sharma & Shastry and *Rosa clinophylla* Thory var. *glabra* S.C. Ghora & G. Panigrahi are the wild relatives of rice and rose, respectively, are the common species of this area.

Faunal diversity in wetlands:

Wetland faunal diversity includes several species of aquatic birds, fishes, amphibians, reptiles, molluscas etc (Plate I). Detailed report on such faunal diversity from this region is not available. The present study recorded many important waterfowls from these wetlands among those Farraka barrage (Kaliachak 2), Nayabandh beel (Habibpur), Chatral – Gabgachi beel complex (Engraz bazaar), Sanak beel (Harischandrapur 2), Barbilla beel (Ratua 2), Boalia beel (Chanchal 1), Belatuli beel (Old Maldah) and Madhaipur beel (Old Maldah) are important. These natural wetlands are very rich in fish faunas as these are very old and with dense aquatic vegetation in most of the areas. But, almost every year, during floods fishes from nearby ponds and from rivers enter these beels. Some of the common fishes like *Catla catla, Labeo rohita, Chana punctata, Clarias batrachus, Heteropneustes fossilis, Oreochromis mossambicus, Trichogastes fasciatus, Puntius sarana, Channa striatus, Burbus tinto* (titputi), *Puntius ticto, Anabas testudineus, Collisa fasclata* (Khalisa) etc. are very common.

The rich avifauna also attracts the bird watchers and hunters of the region equally. During September to February, several species of waterfowls like large Pelican, Adjuvent, Heron, Stork, Kingfisher and some species of ducks remain there in large numbers. Few common avifauna includes *Aythya fuligula* (Tufted Duck), *Anas acuta* (Northern pintail), *Anas clypeata* (Northerm Shoveler), *Anas strepera* (Gadwall), *Anas anser, Ardeola grayii, Ardea cinerea* (Indian Pond Heron), *Phalacrocorax niger, Phalacrocorax carbo, Leptoptilos javanicus* (Lesser Adjutant Stork), *Leptoptilos dubius* (Greater Adjutant Stork), *Anastomus osciyans* (Asian Openbill), *Helcyon capensis* (Stork Billed Kingfisher), *Alcedo atthis, Alcedo meninting, Ergetta garzetta* etc. are regular visitors in different wetlands of Maldah.

Other animal groups like some snakes (*Naja naja, Xenochrophis piscator* etc.), lizards (*Varanus benghalensis, V. salvator* etc.) tortoise like *Melanochelys trijuga indopenninsularis* and Moluscas (etc.) are common.

Dependence of local people:

The people of this region depend very much on these wetlands. They use wetland-water for irrigation during dry season. Several fishermen catch fishes from those wetlands to run their life. Several wild plant species, they collect from the wild and are used in various ways like medicine (33 species), food (26 species), fodder (>30 species), building materials, manure (9 species), fuel (8 species), etc (Chowdhury & Das, 2009).

Marketing of numerous species of plants, fishes and hunted birds from these wetlands are commonly sold in different local markets. Most of the wetlands are also used for the cultivation of Makhana (*Euryale ferox* Salisbury), water-chestnut (*Trapa natans* var. *bispinosa* (Roxburgh) Makino), Lotus (*Nelumbo nucifera* Gartner) for their market demand. The wetlands are the preferred areas for retting jute.

Status of conservation:

The continuous population growth and urbanization are gradually destroying the wetlands and, thereby biodiversity of the area is badly affected. The threats facing these wetlands are of two types. Natural threats include reduction of wetland depth by deposition of dead plant parts and siltation; and anthropological pressure includes rapid urbanization, industrialization, sewage discharge, cultivation etc. Both the natural and anthological pressure rapidly reducing the wetland depth and poisoning the water. All these facts are rapidly destroying the important wetland ecosystem. The wetlands of Maldah district deserve immediate protection to conserve the biodiversity in their natural conditions. To protect this wealth Government, NGOs and public should work together. For this public awareness is to be

generated fast through different public-contact programs, pin-pointed research activities, for mulation of proper management strategies, and strict implementation of existing conservation related rules of the land. However, declaring some of these wetlands as Ramsar Sites may improve the situation.

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Begomovirus causing leaf curl disease in tomato (*Lycopersicon esculentum* L.) in sub-Himalayan West Bengal, India.

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Abstract
Tomato (*Lycopersicon esculentum* L.) is an extensively cultivated vegetable crop in India. In the year 2009, a survey was conducted to find leaf curls of tomato in different locations of sub-Himalayan West Bengal, India. During the survey a severe leaf curl disease was observed. The characteristic disease symptoms (puckered leaves) and presence of whitefly (*Bemisia tabaci*) population indicated the possibility of begomovirus infection. Total DNA was extracted from the infected samples and PCR was carried out using begomovirus specific primers. An amplicon of expected size (~1280 bp) was found when PALIc1960 and PARIv722 were used as primers in agarose gel electrophoresis. The PCR Amplicons of two samples (collected from two different places of present study area) were cloned and sequenced (GenBank accession nos. HM856626 and HM856627). The sequence data analysis of partial coat protein gene (AV1), full replication enhancer protein gene (AC3) and partial transcription activator protein gene (AC2) of 831nt revealed highest 98% similarities with several isolates of *Tobacco curly shoot virus* (TbCSV) at both nucleotide and amino acid levels. The phylogenetic analysis also showed close relationship of the present isolates with different variants of TbCSV. Based on highest sequence similarities and closest relationships with TbCSV the viruses (present in infected tomato plants) were considered as *Begomovirus*. Transmission of the virus in tomato could not be done by sap transmission procedure. In experimental insect transmission tests, test plants showed symptoms very much like the natural symptoms. Artificial transmission was confirmed by comparing the PCR Amplicons raised from the experimentally infected plants.

Key words: Tomato, *Begomovirus*, *Bemisia tabaci*.

Introduction

Tomato leaf curl diseases (TLCDs) occur in many tomato producing regions of the world. The disease is characterized by severe leaf curling, shrinking of tomato leaves and stunted plant growth. TLCD is caused by geminiviruses (genus *Begomovirus*, family *Geminiviridae*) and is transmitted by whitefly. Geminiviruses are a major constraint for the successful cultivation of tomato in tropic and subtropics (Czosnek, 1988; Hong and Harrison, 1995). Several geminivirus species infecting tomatoes from old world have been characterized (Padidam *et al.*, 1995).

Vasudeva and Samraj (1948) for the first time reported the occurrence of tomato leaf curl disease in India. Presently TLCD is a serious problem for tomato-growing regions in India. On the basis of biological and molecular characteristics attempts have been made to characterize the causal agent(s) of the disease. A number of species or strains of tomato leaf curl geminiviruses have been reported to cause TLCDs in India (Vasudeva and Samraj, 1948; Sastry and Singh, 1973; Muniyappa and Saikia, 1983; Saikia and Muniyappa, 1989; Harrison *et al.*, 1991; Reddy *et al.*, 2005; Kirthi *et al.*, 2002; Paximadis *et al.*, 2001; Ramappa *et al.*, 1998). Reddy *et al.* (2005) reported *Tomato leaf curl New Delhi virus* (ToLCNDV) from Assam and West Bengal (Kolkata). Beside *Tomato leaf curl virus*, TLCDs of tomato are also caused by *Tobacco curly shoot virus* (TbCSV) and *Tobacco leaf curl Yunnan virus* (TbLCYNV) in China (Li *et al.*, 2004).

In tropical and subtropical climate zones, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) is an important insect pest. *B. tabaci* provokes direct feeding damage but also causes considerable indirect damage as a vector of numerous geminiviruses (Credi *et al.*, 1989) such as *Tomato*

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yellow leaf curl virus, a threatening virus of tomato (Markhan *et al.*, 1995; Moriones and Navas-Castillo, 2000; Pico *et al.*, 1996).

The present study deals with the begomovirus, a causal pathogen of tomato leaf curl disease.

MATERIALS AND METHODS

Field observation and virus source

A survey of tomato growing fields of sub-Himalayan West Bengal was conducted during winter season of 2009 and 2010 for observation of viral disease problems of tomato plants. The naturally infected plants showing symptoms of tomato leaf curl diseases (TLCDs) were collected from the fields and were tested by polymerase chain reaction (PCR) using universal begomovirus specific primer pair. Viruses were maintained in tomato plants grown in separate net houses in experimental garden, Department of Botany, University of North Bengal, Siliguri.

Transmission by whitefly and symptom development

Virus-free white flies were used as vectors in transmission experiment and insect transmission was done following the technique as described by Ghanem *et al.* (2001). About twenty insects were allowed to feed on infected tomato plants in an insect proof cage for 24 hours (acquisition access period). After 24 hours all the 20 insects were transferred to healthy plants, duly covered by a fine mosquito net. The insects were allowed to feed for 72 hours on healthy tomato plants. After 72 hours all the insects were removed carefully from the plant by shaking the plant and the insect-free plant was left for symptom development up to 60 days. Symptoms started appearing after 20 days but severe symptom development was found after 30 days. However, the presence of virus was further confirmed by PCR amplification of the viral coat protein (partial) from the artificially infected (insect-transmitted) leaves of tomato.

Extraction of total DNA of virus infected plants

Total DNA were extracted from the Infected and healthy plants. Cetyl trimethyl ammonium bromide (CTAB) method of Dellaporta *et al.* (1983) modified by Sharma *et al.* (2003) was followed for extraction of DNA. All extracted DNAs were diluted 10-fold in sterile distilled deionised water just before PCR amplification.

Polymerase chain reaction (PCR)

The forward and reverse Rojas universal primers (Rojas *et al.*, 1993) PALIc1960 (5'ACNGGNAARACNATGTGGGC3') and PARIv722 (5'GGNAARATHTGGATGGA3') were used for amplification of position "722" within the CP gene through the AC2 and AC3 gene to position ~1960 of DNA-A segment. Amplification of the DNA was performed in a volume of 25µl of reaction mixture containing 2µl DNA template, 2.5µl 10x Taq DNA buffer B (Genie, Bangalore), 1.5µl 1.5mM $MgCl_2$ (Genie, Bangalore), 0.5µl 2.5mM dNTPmix (Genie, Bangalore), 0.5µl 1 pmol each forward and reverse primers (Sigma, USA) and 1µl 0.5U/µl Taq DNA polymerase (Genie, Bangalore). The amplification was carried out using a Gene Amp 2400 thermal cycler PCR system (Perkin Elmer). Amplification programme consisted of one initial cycle of denaturation at 94ÚC for 1 min, annealing at 52ÚC for 1min 30sec and extension at 72ÚC for 2min. After the initial cycle, 40 cycles of PCR were performed of denaturation (94ÚC for 50sec), annealing (at 52ÚC for 45sec) and extension (at 72ÚC for 1min 30sec). After that amplification programme was continued for 10 minutes at 72ÚC. Amplification products were electrophoresed at 5V/cm through 1.5% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) electrophoresis buffer, and visualized under UV transilluminator following ethidium bromide staining of the gel (30min in 1µg/ml ethidium bromide).

DNA sequencing and phylogenetic analysis

The amplified products of expected size were either directly sent for sequencing or cloned into the pGEM T-Easy vector (Promega, Madison, USA). The sequencing was done from DNA sequencing service, Genie, Bangalore. The sequences were submitted to GenBank with proper annotations. The accessions of the GenBank have also been received. The sequences were compared to the equivalent sequences from a range of other geminiviruses present in GenBank and have been

mentioned in details in elsewhere in the present article. Multiple sequence alignment was carried out using the software clustalW in MEGA *version* 4 (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004).

Results and Discussion

Field observation and virus isolation

Infected tomato plants were observed in ten different places of sub-Himalayan West Bengal during December 2009 to February 2010. Considerable damages were found to occur in two widely cultivated tomato varieties (Pusa Ruby and Rocky variety). Out of ten infected plants two tomato plants (from Siliguri and Haldibari) with TLCDs (Figure 1) showed PCR positive results. PCR positive plants were selected for further analysis.

Figure1: Tomato plants showing TLCDs in field from Siliguri (a) and Haldibari (b). Healthy tomato plant in field (c)

Polymerase chain reaction (PCR)

Whole DNA of infected samples were extracted and specific genes of viruses were amplified in PCR. For PCR experiments specific primers were used and molecular weight of the amplicons were determined by using standard DNA-molecular weight markers (ladders) on agarose gel. The size of the amplified DNAs (by primers PALIc1960 and PARIv722) were ~1280bp which matched with the molecular weight as suggested by Reddy *et al.* (2005). The DNA samples showed presence of virus, were subjected to cloning and finally clones were sent for sequencing. The results of some amplified samples along with DNA-molecular weight markers have been presented in Figure 2. Lanes designated as M contained DNA ladders, Lanes (L1 and L2) contained PCR product of healthy plants (control) and Lanes (L3-L6) contained PCR amplified products.

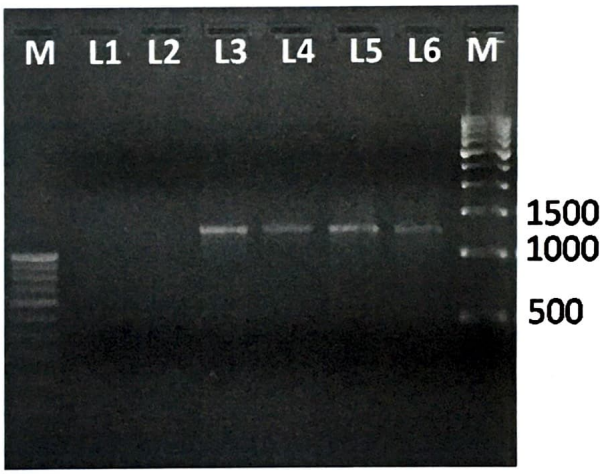

Figure 2: 1.5% agarose gel electrophoresis showing the PCR products of primer PALIc1960/PARIv722 of tomato samples from Siliguri (L3) and Haldibari (L4). L5 and L6 represent the PCR product from insect transmitted tomato samples. L1 and L2 are control. M: DNA Ladder 500 and 1000bp.

Sequence analysis

Sequence data revealed that the PCR product of Siliguri (SILIGURI-1) consisted of 831 nucleotides spanning 3'-terminal 375 nucleotides of CP gene (125aa), the entire 405 nucleotides of AC3 coding region (135aa) and the 3'-terminal 315 nucleotides of AC2 gene (105aa) and the PCR products of HALDI-1 consisted of 741 nucleotides. BLAST

analysis revealed that the new viruses are closely related to *Tobacco curly shoot virus*. The highest percent sequence identities (presented in table-1) for nucleotide sequence of SILIGURI-1 sample was 98% with *Tobacco curly shoot virus*, China (Accession no. GU001879). Similarly, HALDI-1 also showed 98% sequence identities with another *Tobacco curly shoot virus*, China (Accession no. AF240675). The amino acid sequence of complete AC3 gene showed 99% sequence similarity with *Tobacco curly shoot virus* (Figure 3). The Accession numbers for the samples SILIGURI-1 and HALDI-1 are respectively HM856626 and HM856627.

Phylogenetic analysis

In phylogenetic tree (Figure 4), the isolates from Haldibari and Siliguri (HM856626 and HM856627) formed cluster with three *Tobacco curly shoot virus* (Accession nos. GU001879, AJ971266 and GU199583) reported from China with bootstrap value ranging from 83% to 96%. BLAST analysis and Phylogenetic analysis revealed that the two viruses are closely related to *Tobacco curly shoot virus*.

Tomato leaf curl samples were collected from several places of sub-Himalayan West Bengal. DNA extracts of field samples were amplified using begomovirus specific primer pairs. The amplified sequences were compared with the near-similar sequences present in the GenBank. Similar studies were also done by Reddy *et al.* (2005) but they included only three main locations (Kolkata, Maligaon and Patna) in eastern India. From our study it is evident that TLCDs were produced by the begomoviruses. Reddy *et al.* (2005) reported two other begomoviruses from tomato plants of Patna and Kolkata. However, those two viruses (PepLCV and ToLCGV) were not reported from the present tomato samples studied. Presence of *Tobacco curly shoot virus* (TbCSV) and *Tobacco leaf curl virus* (TbLCV) in tomato causing leaf curl diseases have also been reported by Li *et al.* (2004) from China.

In the family *Geminiviridae* CP genes are most

Table 1: The percent identities between the partial AV1 gene, complete AC3 gene and partial AC2 gene regions of isolate SILIGURI-1 DNA associated with the tomato disease and those of 10 most closely related geminiviruses.

Accession No. of geminiviruses	Locus name	Percent identities [with Partial AV1, complete AC3 and Partial AC2 of isolate SILIGURI-1 DNA (Accession No. HM856626)]
GU001879	*Tobacco curly shoot virus*-[SC118], complete genome	98
AJ971266	*Tobacco curly shoot virus*-[Y282] complete genome, isolate Y282	98
GU199583	*Tobacco curly shoot virus* clone 20-9, complete genome	98
AJ437618	Ageratum enation virus complete genome	97
AY738103	*Papaya leaf curl virus* from India	95
AF188481	*Tomato leaf curl Bangladesh virus* complete genome	94
EU194914	*Euphorbia leaf curl virus* isolate Pusa Bihar, complete genome	94
EF175733	*Radish leaf curl virus* segment A, complete sequence	94
FN543112	*Croton yellow vein virus*, complete genome, clone 1	93
GQ183868	*Sunn hemp leaf distortion virus* [India: Barrackpore:2008] segment DNA A, complete sequence	92

(a)

```
HM856626 1    NSVMFFLVRDRRPVDKPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTGGQYA 60
              NSVMFFLVRDRRPVDKPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTGGQYA
ADB19845 133  NSVMFFLVRDRRPVDKPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTGGQYA 192

HM856626 61   SKEQALVKKFVRVNNYVVYNQQEAGKYENHSENALMLYMACTHASNPVYATLKIRIYFYD 120
              SKEQALVKKFVRVNNYVVYNQQEAGKYENHSENALMLYMACTHASNPVYATLKIRIYFYD
ADB19845 193  SKEQALVKKFVRVNNYVVYNQQEAGKYENHSENALMLYMACTHASNPVYATLKIRIYFYD 252

HM856626 121  SVTN                                                        124
              SVTN
ADB19845 253  SVTN                                                        256
```

(b)

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HM856626 1    MDSRTGELITAAQAENGVYIWEIQNPLYFKITEHQNRPFLMKEDIITIQIQFNYNLRKAL 60
              MDSRTGE+ITAAQAENGVYIWEIQNPLYFKI EHQNRPFLMKEDIITIQIQFNYNLRKAL
ADB19846 1    MDSRTGEVITAAQAENGVYIWEIQNPLYFKIIEHQNRPFLMKEDIITIQIQFNYNLRKAL 60

HM856626 61   GVHKCFLVYRIWMTSQPQTGRFLRVFKTQVFKYLNNLGIISINNVIRAVDRVLWDVLEHI 120
              GVHKCFLVYRIWMTSQPQTGRFLRVFKTQVFKYLNNLGIISINNVIRAVD VLWDVLEHI
ADB19846 61   GVHKCFLVYRIWMTSQPQTGRFLRVFKTQVFKYLNNLGIISINNVIRAVDHVLWDVLEHI 120

HM856626 121  VYVDQSYSIKFNIY                                              134
              VYVDQSYSIKFNIY
ADB19846 121  VYVDQSYSIKFNIY                                              134
```

(c)

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HM856626 6    CGCSYFIALACHDHGFTHRGTHHCSSSREWRVYLGDSKSPLFQDNRAPEPSISHERRHNH 65
              CGCSYFIALACHDHGFTHRG+HHCSSSREWRVYLGDSKSPLFQDNRAPEPSISHERRHNH
ADB19847 36   CGCSYFIALACHDHGFTHRGSHHCSSSREWRVYLGDSKSPLFQDNRAPEPSISHERRHNH 95

HM856626 66   NPNTVQLQPAESSGSAQVFSSLPNLDDFTASDWSFLKGL                     104
              +PNTVQLQPAESSGSAQVFSSLPNLDDFTASDWSFLKGL
ADB19847 96   HPNTVQLQPAESSGSAQVFSSLPNLDDFTASDWSFLKGL                     134
```

Figure 3: The difference of amino acid sequences between TbCSV isolate SILIGURI-1(Accession no. HM856626) partial gene and *Tobacco curly shoot virus*, China (Accession no. GU001879). The region corresponding to the partial AV1 gene (a), complete AC3 gene (b) and partial AC2 gene (c) is indicated on the alignment. Amino acids are presented with the single-letter code. Identical amino acids were shown with black letters and non identical amino acids are shown with red letters. The consensus amino acid sequence between the two sequences is shown under the alignment.

conserved (Wyatt and Brown, 1996). Identification of virus and their geographic and vector relationship can be correlated with CP sequences (Brown *et al.*, 2001). CP gene partial sequence provides provisional virus identification. For establishing new begomovirus species and for definite classification complete sequence of DNA-A is necessary (Faquet and Stanley, 2005). Many viruses and viral variants were found in *Begomovirus*, and begomoviruses are classified on the basis of genome sequences, especially DNA-A sequence (Harrison *et al.*, 1993). According to Padidam *et al.* (1995) two species of a virus should have less than 90% nucleotide sequence identity and two strains of a virus must have sequence identity greater than 90%. The nucleotide sequences of isolate SILIGURI-I and HALDI-1 showed highest 98% sequence similarity with Chinese *Tobacco curly shoot virus* (TbCSV). The nucleotide sequence of the SILIGURI-1 isolate comprises partial coat protein gene (AV1), replication enhancer protein gene (AC3) and partial transcription activator protein gene (AC2) as reported by Yan *et al.* (2002). In phylogenetic tree the isolate made a cluster with TbCSV (Chinese isolate). In the present study, the causal pathogen was provisionally determined to be a *Begomovirus* through cloning, nucleotide sequence analysis and phylogenetic analysis. This is the first record of a *Begomovirus* infecting tomatoes of sub-Himalaya West Bengal, India. The

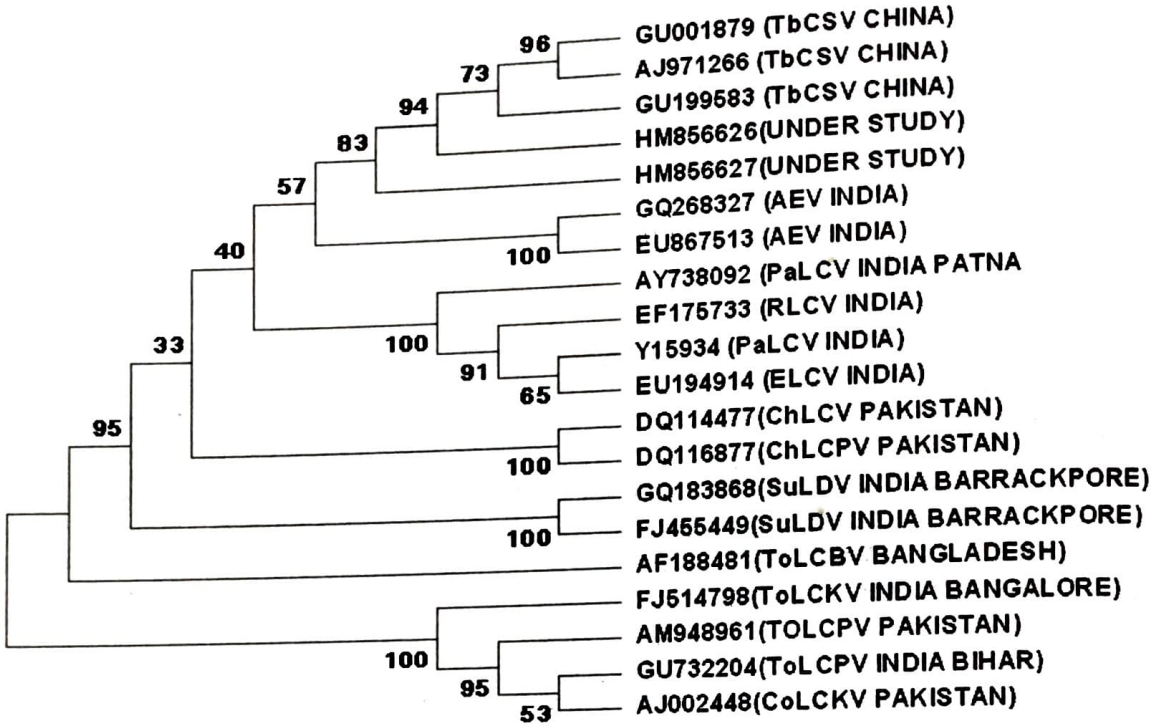

Figure 4: Most parsimonious tree showing the relationship of Partial coat protein (AV1), replication enhancer protein (AC3) and partial transcription activator protein (AC2) gene (HM856626 and HM856627) of isolate SILIGURI-1and HALDI-1 with published begomovirus sequences from GenBank. Numbers at nodes indicate the bootstrap percentage scores out of 1000 replicates.

sequence information in this study would be helpful in understanding and management of the new pathogen of TLCDs.

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Effect of copper on seed germination, root elongation and shoot elongation of seedlings of commercially cultivated tea varieties

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Abstract
Tea (*Camellia sinensis* L. (O.) Kuntze) is an economically important plantation crop of India but is prone to attack by several fungal pathogens. Copper based fungicides are being used for decades to control fungal disease in tea which may lead to accumulation of copper in the soil. The aim of the present work was to investigate toxic effect of Cu^{2+} on seed germination, growth and morphological changes in tea seedlings. Different concentration of copper sulphate was applied on three different commercially cultivated tea varieties (TS-462, TS-520 and TS-463). The effect of different concentrations of copper (0.5 – 8 mM) on seed germination, root elongation and shoot elongation of the tea plant were evaluated. Percent germination was found to decrease progressively with increasing concentrations of Cu^{2+}. Maximum reduction of seed germination was showed by TS-463. Several damaging effects such as reduced root hair proliferation, structural deformation and reduction in length of root and shoot were observed when the germinated seedlings were allowed to grow at higher concentrations of copper solutions. Decrease in the dry mass of both root and shoot were also recorded. The results showed that excess copper have negative effect on germination of tea seeds and subsequent growth of the seedlings.

Key words: Tea seedlings, copper, germination, growth.

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is the most important and popular non-alcoholic beverage. Seeds or vegetative clones are the main sources of tea plant propagation. Successful production of crops is ordinarily associated with healthy shoot and root growth. Tea plants are prone to attack by several fungal pathogens which cause diseases such as blister blight, brown blight, grey blight, black rot, pink disease and thread blight leading to major economic losses (Tripathi 2006). To control the various diseases, copper-fungicides are used excessively in tea gardens of north-east India including Assam and sub-Himalayan West Bengal (Barua 1988, Singh 2005). The fungicides that are used most commonly include basic copper sulphate, Bordeaux mixture, Bicoxy (copper oxychloride 50% WP) and various customized formulations of copper sulphate and copper oxychloride (Worthing 1983, Sanjay *et al.* 2008). Agricultural practices with a long history of copper fungicide application have resulted in high levels of copper in soil that has affected a large portion of agricultural land (Brun *et al.* 1998).

Copper is a constituent of certain enzymes such as cyctochrome oxidase, polyphenol oxidase, tyrosinase, amine oxidase and superoxide dismutase. Thus copper in trace amount is essential for various metabolic processes in the plant but at higher concentrations it causes physiological stress (Dat *et al.* 2000, Saha *et al.* 2012). Copper content of whole plants exceed 20 ppm (on dry weight basis) and this value is most often considered to indicate the threshold limits (Singh *et al.* 2007). High levels of Cu^{2+} application to soil and leaves have been found to disrupt normal plant growth. The toxic effect of Cu^{2+} has been attributed to the redox nature of this element that induces over-production of reactive oxygen species which in turn interferes with the photosynthetic electron transport and damages the cell ultrastructure (Babu *et al.* 2001, Yruela 2005 and Quian *et al.* 2009).

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Applications of high levels of Cu^{2+} usually inhibit root growth and shoot production (Wisniewski and Dickinson, 2003, Peralta *et al.* 2000, Lombardi & Sebastiani, 2005). Sonmez *et al.* (2006) reported an increasing reduction in total yield, fruit number, dry root weight and plant height with increasing levels of Cu^{2+} application to soil and leaves. Increased copper concentrations have been reported to reduce percent germination, root and shoot elongation in maize (Hunter 1981), tomato (Mazhoudi *et al.* 1997), citrus (Alva *et al.* 2000), wheat (Singh *et al.* 2007), rice (Chen *et al.*2000, Lidon & Henriques, 1992) etc.

An initial survey of several tea gardens of North Bengal has revealed that copper-fungicides are extensively used in the tea gardens of the Dooars and Terai region and also in the Hilly region of West Bengal. This extensive use of copper over a long time has necessitated research on effect of copper on tea plants. The aim of the present work was to investigate toxic effect of Cu^{2+} on seed germination, growth and morphology of tea seedlings.

Materials and Methods

Plant Materials and Chemicals: Tea seeds of three different biclonal seed stocks (viz. TS-520, TS-463 and TS-462) were procured from Gayaganga Tea Estate, Siliguri, India. Sixteen different concentrations (0.5–8.0 mM) with 0.5 mM increments of copper solutions were prepared freshly and used for application. Control sets were treated with distilled water.

Application of copper and germination of tea seeds: To determine the percent germination in the treated and untreated seeds of TS-520, TS-463 and TS-462, metal application procedure as described by Munzuroglu and Geckil (2002) was followed with some modifications. Seed surfaces were sterilized with 0.01% $HgCl_2$, washed twice with sterile distilled water and sowed in earthen pots (6 cm diameter) containing moist sterilized sand. The pots were placed in dark in growth chamber at 25°C. Following rupturing of seeds which occurred after 20 d, the seeds were placed in sand and wetted with 4 ml of Cu^{2+} solution of each concentration. The pots were kept at 25°C in the growth chamber and the germination was noted every 24h. Seeds were considered to be germinated at one mm of radical emergence and percent germination was calculated. The length of the shoots as well as roots of each variety was recorded at 15, 18, 21, 24 and 27d after treatment and deformities, if any were noted. The dry weight of treated and untreated shoots and roots of the tested varieties were measured after 27d of treatment. This time duration was followed based on observations obtained during preliminary studies which showed that this time was necessary to test appropriate inhibitory effects. A set of plants (exposed to 6.5 mM Cu^{2+} concentration) were allowed to grow until 60 days for observing the progressive deformations in the roots over a longer period.

Results and Discussion

Figure 1 summarizes the effect of different concentrations of copper on seed germination of three tested tea seed varieties of north east India. In general there was a progressive reduction in seed germination as metal concentration increased in all the tested varieties. Minimum seed germination was recorded when 8 mM copper solution was applied. Germination percentage was found to be lowest in TS-463 (17%) and highest in TS-462 plants (25%). Peralta *et al.* (2000) investigated the individual effects of several doses of heavy metals on the growth of live alfalfa plants using solid media. They used 0, 5, 10, 20 and 40 ppm doses for experiment and according to them 20 ppm concentration of Cu^{2+} significantly affected

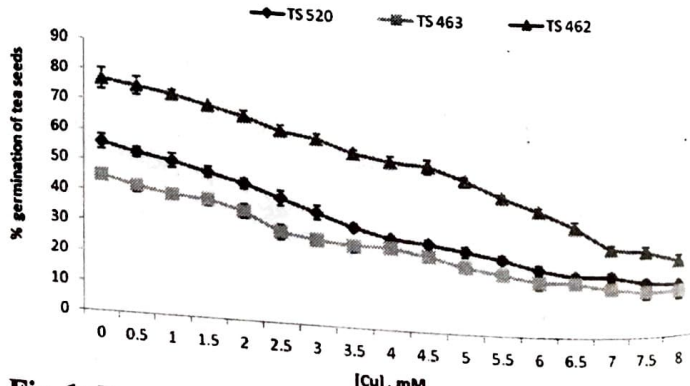

Fig. 1: Percent germination of seeds of TS-520, TS-463 and TS-462 at different concentrations of copper solution.

the seed germination and plant growth. Claire *et al.* (1991) obtained similar results in a study using copper and other heavy metals on cabbage, lettuce, millet, radish, turnip, wheat and alfalfa plants. Singh *et al.* (2007) observed that the germination (60%), plumule and radicle length, and number of lateral roots of wheat decreased with increase in copper concentration (5, 25, 50 and 100 mgl^{-1}) after 14th and 21st day of treatment.

Both root and shoot elongation was hampered with increase in the concentration of Cu^{2+} for all the three varieties. Maximum reduction in length of shoot (91%) was observed in TS-463 variety followed by TS-520 (86%) and TS-462 (68%) (Tables 1, 2, 3). Root elongation was most affected in TS-462 (96%) followed by TS- 463 (91%) and TS-520 (84%) (Tables 4, 5, 6). Higher concentration of Cu^{2+} (>6.5 mM) showed several damaging effects such as reduced root hair proliferation, reduction in the number of root hairs, blacking of the root tips, stunted growth, deformed root and shoot structure and substantial reduction in the length of the root and shoots in all tested varieties (Fig. 2). Sheldon and Menzies (2004) observed that excess copper in *Chloris gayana* seeds caused damage to the cuticle on the main root, reduction in the number and length of root hairs on the main root and damage to the root meristem. According to Peralta *et al.* (2000), Cu (II) exerts detrimental effects at the dose of 40 ppm and 10 ppm causing a shoot and root elongation reduction of 70.0%, 54% respectively in (*Medicago sativa*). Ali *et al.* (2006) observed that root treated with 50 µM copper resulted in 52% and 89% growth inhibition after 20 & 40 days of treatment respectively in *Panax ginseng*. Manivasagaperumal *et al.* (2011) reported that 100-200 mg/kg concentration of copper reduces the growth of the shoots and roots of *Vigna radiata*.

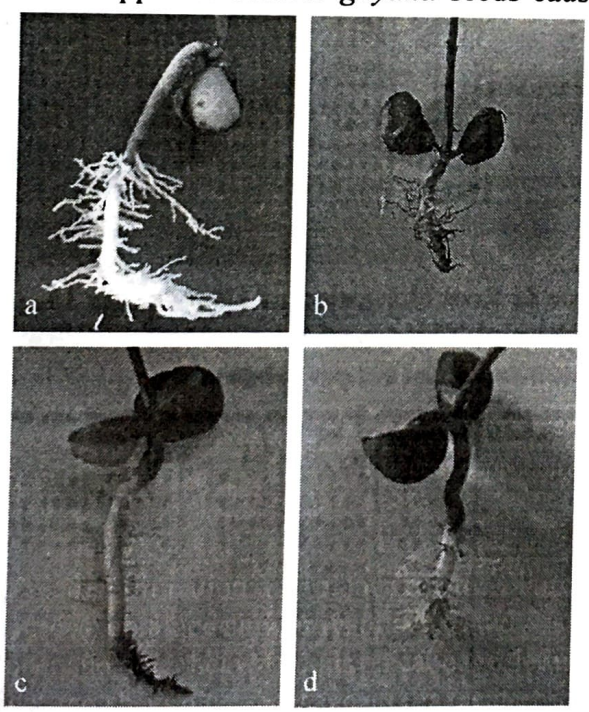

Fig. 2: Root deformations observed when tea seeds were allowed to germinate in high Cu^{2+} concentrations (6.5 mM) for 60 days. a: Control, b: TS-463; c: TS-520; d: TS-462.

Effect of high concentrations of copper on dry weight of shoots and roots were also tested and the results are summarized in Fig 3 & 4. Dry weight of both shoots and roots were decreased with increasing concentration of Cu^{2+}. Dry weight of roots reduced to 0.042 mg, 0.013 mg and 0.017 mg in tea seedlings treated with 8 mM concentration

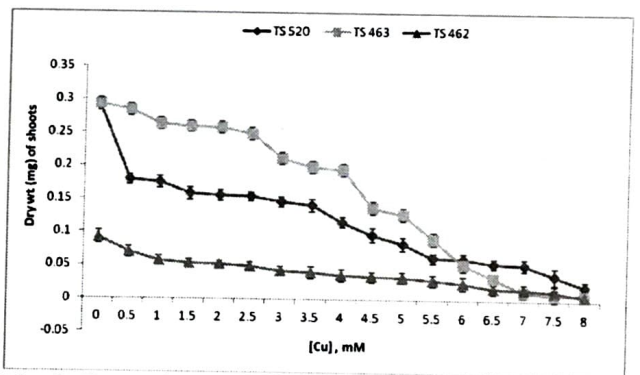

Fig. 3: Dry weight of shoots of TS-520, TS-463 and TS-462 at different concentrations of copper solution after 27 days of treatment

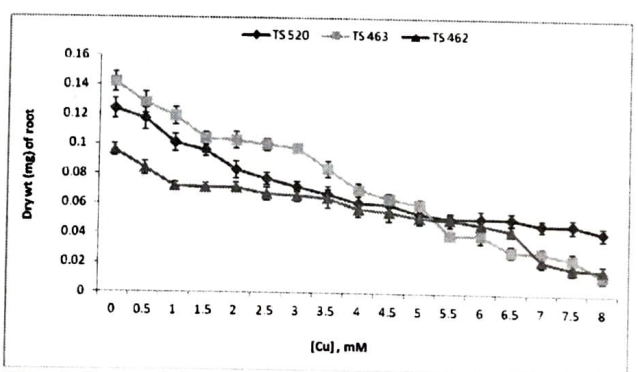

Fig. 4: Dry weight of root of TS-520, TS-463 and TS-462 at different concentrations of copper solution after 27 days of treatment

Table 1: Shoot elongation of TS-520 after 15, 18, 21, 24 and 27 days of treatment with different concentrations of copper solution

Concentration of $CuSO_4$ (mM)	Shoot length variety (in cm) was measured at different days after treatment in TS520				
	15d	18d	21d	24d	27d
0	9.2 ± 0.5	10.4 ± 0.45	11.2 ± 0.3	13.5 ± 0.25	15.2 ± 0.3
0.5	6.7 ± 0.3	8.4 ± 0.3	9.6 ± 0.3	10.3 ± 0.5	10.6 ± 0.45
1	6.5 ± 0.4	7.9 ± 0.2	8.9 ± 0.5	10.1 ± 0.65	10.5 ± 0.35
1.5	6.2 ± 0.4	7.2 ± 0.3	8.9 ± 0.2	10.1 ± 0.45	10.5 ± 0.1
2	5.7 ± 0.25	7.2 ± 0.15	8.7 ± 0.3	9.2 ± 0.4	9.9 ± 0.5
2.5	5.2 ± 0.2	6.3 ± 0.5	7.8 ± 0.15	8.8 ± 0.4	9.1 ± 0.15
3	4.7 ± 0.3	6.3 ± 0.4	7.3 ± 0.4	8.3 ± 0.5	9 ± 0.25
3.5	4.6 ± 0.3	6.2 ± 0.4	7.1 ± 0.3	8.3 ± 0.55	8.8 ± 0.5
4	4.6 ± 0.3	5.8 ± 0.4	6.7 ± 0.4	7.2 ± 0.15	8.1 ± 0.2
4.5	4.6 ± 0.2	5.5 ± 0.2	6.4 ± 0.5	6.9 ± 0.6	7.7 ± 0.3
5	3.6 ± 0.2	4.9 ± 0.7	5.2 ± 0.2	6.3 ± 0.6	6.5 ± 0.4
5.5	3.5 ± 0.3	4.2 ± 0.5	5.2 ± 0.2	5.4 ± 0.25	6.2 ± 0.4
6	2.1 ± 0.1	3.6 ± 0.4	4.8 ± 0.45	5.3 ± 0.2	5.7 ± 0.4
6.5	2.1 ± 0.45	3.6 ± 0.2	4.2 ± 0.2	4.7 ± 0.1	5.1 ± 0.4
7	1.5 ± 0.45	3.1 ± 0.3	3.8 ± 0.2	4.2 ± 0.3	4.6 ± 0.35
7.5	1.5 ± 0.1	2.7 ± 0.3	3.2 ± 0.3	4.2 ± 0.2	4.5 ± 0.3
8	1.2 ± 0.3	1.4 ± 0.3	1.6 ± 0.2	1.8 ± 0.5	2 ± 0.4

Table 2: Shoot elongation of TS-463 after 15, 18, 21, 24 and 27 days of treatment with different concentrations of copper solution

Concentration of $CuSO_4$ (mM)	Shoot length (in cm) was measured at different days after treatment in TS-463				
	15d	18d	21d	24d	27d
0	5.2 ± 0.5	6.5 ± 0.3	7.5 ± 0.3	9.8 ± 0.3	11.5 ± 0.7
0.5	5.1 ± 0.3	6.3 ± 0.1	7.1 ± 0.2	9.5 ± 0.45	11.5 ± 0.55
1	4.4 ± 0.3	6 ± 0.4	6.8 ± 0.1	9.2 ± 0.45	11.1 ± 0.6
1.5	4.3 ± 0.5	5.6 ± 0.4	6.4 ± 0.2	9.2 ± 0.2	10.5 ± 0.5
2	4.2 ± 0.2	5.3 ± 0.2	6.3 ± 0.1	9.2 ± 0.5	10.2 ± 0.5
2.5	4.1 ± 0.4	5.2 ± 0.2	6.3 ± 0.2	7.7 ± 0.5	10.1 ± 0.2
3	3.7 ± 0.4	5.1 ± 0.25	6.1 ± 0.2	7.7 ± 0.4	9.4 ± 0.5
3.5	3.3 ± 0.2	4.2 ± 0.1	5.9 ± 0.1	7.5 ± 0.4	8.5 ± 0.5
4	3.1 ± 0.3	3.9 ± 0.4	5.9 ± 0.1	7.1 ± 0.4	8.3 ± 0.2
4.5	3.1 ± 0.2	3.7 ± 0.2	4.9 ± 0.2	6.9 ± 0.3	7.3 ± 0.2
5	2.1 ± 0.2	3.2 ± 0.1	4.3 ± 0.1	6 ± 0.3	6.3 ± 0.25
5.5	1.8 ± 0.2	2.9 ± 0.15	4.2 ± 0.1	5.8 ± 0.3	6.2 ± 0.1
6	1.3 ± 0.2	2.8 ± 0.1	4 ± 0.2	4.8 ± 0.3	5.1 ± 0.1
6.5	1.2 ± 0.15	1.5 ± 0.2	1.9 ± 0.1	2.3 ± 0.2	2.7 ± 0.1
7	1 ± 0.1	1.2 ± 0.2	1.8 ± 0.1	2.1 ± 0.1	2.5 ± 0.1
7.5	0.4 ± 0.1	1.1 ± 0.15	1.3 ± 0.1	1.9 ± 0.15	2.1 ± 0.1
8	0.3 ± 0.2	0.6 ± 0.45	0.7 ± 0.1	0.9 ± 0.25	1 ± 0.3

Table 3: Shoot elongation of TS-462 after 15, 18, 21, 24 and 27 days of treatment with different concentrations of copper solution

Concentration of $CuSO_4$ (mM)	Shoot length (in cm) was measured at different days after treatment in TS-462				
	15 d	18 d	21d	24d	27d
0	4.4 ± 0.5	4.9 ± 0.15	5.5 ± 0.3	6.1 ± 0.1	7 ± 0.2
0.5	4.1 ± 0.4	4.8 ± 0.2	5.1 ± 0.5	5.9 ± 0.4	6.6 ± 0.2
1	3.9 ± 0.2	4.2 ± 0.5	4.2 ± 0.3	5.3 ± 0.3	6.2 ± 0.3
1.5	2.6 ± 0.1	3.8 ± 0.4	3.9 ± 0.2	4.2 ± 0.2	4.9 ± 0.1
2	2.5 ± 0.2	3.1 ± 0.2	3.8 ± 0.2	4 ± 0.3	4.5 ± 0.2
2.5	2.2 ± 0.4	3.1 ± 0.3	3.7 ± 0.1	4 ± 0.1	4.5 ± 0.4
3	2.1 ± 0.1	3 ± 0.3	3.6 ± 0.4	3.9 ± 0.2	4.3 ± 0.1
3.5	1.8 ± 0.3	2.9 ± 0.6	3.1 ± 0.1	3.7 ± 0.1	4.2 ± 0.1
4	1.4 ± 0.3	2.5 ± 0.3	3.1 ± 0.1	3.7 ± 0.1	4.2 ± 0.1
4.5	1.3 ± 0.1	2.4 ± 0.3	3.1 ± 0.15	3.7 ± 0.4	4.1 ± 0.15
5	1.3 ± 0.2	2.3 ± 0.4	3 ± 0.5	3.1 ± 0.3	3.9 ± 0.1
5.5	1.2 ± 0.1	1.9 ± 0.4	2.5 ± 0.3	2.9 ± 0.3	3.2 ± 0.3
6	1.1 ± 0.1	1.6 ± 0.2	2.1 ± 0.1	2.4 ± 0.2	2.8 ± 0.4
6.5	1.1 ± 0.1	1.6 ± 0.15	1.9 ± 0.1	2.3 ± 0.4	2.8 ± 0.2
7	1 ± 0.1	1.5 ± 0.1	1.9 ± 0.2	2.1 ± 0.15	2.5 ± 0.1
7.5	0.9 ± 0.1	1.5 ± 0.3	1.9 ± 0.25	2 ± 0.2	2.3 ± 0.2
8	0.7 ± 0.1	1.3 ± 0.5	1.8 ± 0.3	2 ± 0.2	2.2 ± 0.1

Table 4: Root elongation of TS-520 after 15, 18, 21, 24 and 27 days of treatment with different concentrations of copper solution

Concentration of $CuSO_4$ (mM)	Root length (in cm) was measured at different days after treatment in TS-520				
	15 d	18 d	21d	24d	27d
0	4.5 ± 0.3	6.8 ± 0.3	7.5 ± 0.6	7.7 ± 0.1	7.8 ± 0.1
0.5	4.3 ± 0.2	5.2 ± 0.3	5.9 ± 0.3	6.2 ± 0.2	6.7 ± 0.1
1	4.1 ± 0.2	5.1 ± 0.1	5.3 ± 0.3	5.7 ± 0.2	6.2 ± 0.2
1.5	4.1 ± 0.2	4.5 ± 0.2	4.8 ± 0.2	5.5 ± 0.6	5.7 ± 0.1
2	3.2 ± 0.1	4.2 ± 0.4	4.5 ± 0.45	5 ± 0.3	5.3 ± 0.1
2.5	3.2 ± 0.15	3.9 ± 0.35	4.3 ± 0.55	4.7 ± 0.2	5.1 ± 0.1
3	3.2 ± 0.2	3.5 ± 0.3	3.9 ± 0.5	4.1 ± 0.1	4.3 ± 0.2
3.5	2.9 ± 0.3	3.1 ± 0.1	3.5 ± 0.3	3.6 ± 0.1	3.8 ± 0.1
4	2.7 ± 0.2	3.1 ± 0.4	3.3 ± 0.4	3.4 ± 0.3	3.8 ± 0.1
4.5	2.7 ± 0.4	2.9 ± 0.4	3 ± 0.3	3.2 ± 0.1	3.5 ± 0.1
5	2.2 ± 0.1	2.8 ± 0.4	3 ± 0.5	3.1 ± 0.25	3.3 ± 0.1
5.5	2.1 ± 0.3	2.3 ± 0.2	2.6 ± 0.2	2.7 ± 0.1	2.9 ± 0.2
6	2.1 ± 0.06	2.3 ± 0.15	2.4 ± 0.1	2.6 ± 0.1	2.8 ± 0.1
6.5	1.5 ± 0.3	1.9 ± 0.3	2 ± 0.1	2.2 ± 0.3	2.5 ± 0.2
7	1.4 ± 0.3	1.7 ± 0.2	2 ± 0.25	2.1 ± 0.3	2.4 ± 0.1
7.5	1 ± 0.06	1.6 ± 0.35	1.8 ± 0.15	1.9 ± 0.2	2.1 ± 0.1
8	0.6 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	1 ± 0.1	1.2 ± 0.1

Table 5: Root elongation of TS-463 after 15, 18, 21, 24 and 27 days of treatment with different concentrations of copper solution

Concentration of $CuSO_4$ (mM)	Root length (in cm) was measured at different days after treatment in TS-463				
	15 d	18 d	21d	24d	27d
0	3.8 ± 0.1	4.5 ± 0.2	6.3 ± 0.3	7.1 ± 0.2	8 ± 0.15
0.5	2.9 ± 0.5	3.1 ± 0.3	3.8 ± 0.3	4.7 ± 0.2	5.1 ± 0.1
1	2.5 ± 0.1	3.1 ± 0.1	3.6 ± 0.3	4.2 ± 0.15	4.9 ± 0.3
1.5	2.1 ± 0.3	2.9 ± 0.5	3.4 ± 0.3	4.1 ± 0.1	4.5 ± 0.3
2	2.1 ± 0.2	2.8 ± 0.6	3.2 ± 0.6	3.7 ± 0.1	4.4 ± 0.15
2.5	1.8 ± 0.4	2.1 ± 0.1	2.9 ± 0.2	3.3 ± 0.3	4.1 ± 0.15
3	1.7 ± 0.1	2.1 ± 0.5	2.8 ± 0.3	3.1 ± 0.15	3.8 ± 0.1
3.5	1.3 ± 0.2	1.9 ± 0.1	2.7 ± 0.1	3 ± 0.2	3.2 ± 0.1
4	1.3 ± 0.25	1.8 ± 0.2	2 ± 0.15	2.5 ± 0.2	2.9 ± 0.15
4.5	1.3 ± 0.2	1.7 ± 0.1	2 ± 0.75	2.4 ± 0.1	2.9 ± 0.15
5	1 ± 0.2	1.6 ± 0.2	1.8 ± 0.1	2.1 ± 0.1	2.8 ± 0.1
5.5	1 ± 0.4	1.4 ± 0.1	1.7 ± 0.2	2.1 ± 0.2	2.5 ± 0.1
6	0.9 ± 0.2	1.1 ± 0.1	1.6 ± 0.2	2 ± 0.1	2.2 ± 0.1
6.5	0.9 ± 0.3	1 ± 0.2	1.3 ± 0.2	1.9 ± 0.2	2.1 ± 0.1
7	0.7 ± 0.1	0.9 ± 0.45	1.1 ± 0.15	1.3 ± 0.1	1.6 ± 0.1
7.5	0.6 ± 0.1	0.8 ± 0.3	0.8 ± 0.1	0.9 ± 0.2	1 ± 0.1
8	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.15	0.7 ± 0.1

Table 6: Root elongation of TS-462 after 15, 18, 21, 24 and 27 days of treatment with different concentrations of copper solution

Concentration of $CuSO_4$ (mM)	Root length (in cm) was measured at different days after treatment in TS-462				
	15 d	18 d	21d	24d	27d
0	3.8 ± 0.1	4.7 ± 0.2	5.2 ± 0.1	5.8 ± 0.3	6.5 ± 0.1
0.5	3.5 ± 0.1	3.8 ± 0.1	4.1 ± 0.1	4.2 ± 0.15	4.4 ± 0.15
1	3.5 ± 0.1	3.8 ± 0.1	4 ± 0.2	4.1 ± 0.2	4.3 ± 0.15
1.5	3.1 ± 0.2	3.1 ± 0.1	3.5 ± 0.1	3.8 ± 0.25	4 ± 0.2
2	2.8 ± 0.1	3 ± 0.2	3 ± 0.1	3.2 ± 0.2	3.5 ± 0.1
2.5	2.8 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	3.1 ± 0.2	3.5 ± 0.2
3	2.6 ± 0.15	2.8 ± 0.1	2.8 ± 0.1	3.1 ± 0.1	3.3 ± 0.1
3.5	2.5 ± 0.15	2.7 ± 0.1	2.8 ± 0.2	3 ± 0.1	3.3 ± 0.15
4	2.5 ± 0.1	2.7 ± 0.1	2.8 ± 0.4	3 ± 0.2	3.2 ± 0.1
4.5	2.4 ± 0.2	2.6 ± 0.2	2.8 ± 0.3	3 ± 0.15	3.2 ± 0.15
5	1.8 ± 0.1	2.1 ± 0.2	2.7 ± 0.1	2.8 ± 0.3	3.2 ± 0.2
5.5	1.5 ± 0.1	2 ± 0.1	2.5 ± 0.2	2.5 ± 0.2	3.1 ± 0.1
6	1 ± 0.1	1.8 ± 0.1	2 ± 0.15	2.1 ± 0.1	2.2 ± 0.1
6.5	0.9 ± 0.1	1 ± 0.15	1.1 ± 0.1	1.4 ± 0.1	1.5 ± 0.15
7	0.8 ± 0.2	1 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.5 ± 0.1
7.5	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
8	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.05	0.2 ± 0.1	0.2 ± 0.1

of Cu^{2+} in comparison to control which recorded 0.124 mg, 0.142 mg and 0.096 mg root dry weight of TS-520,TS-463 and TS-462 respectively. Dry weight in shoots also showed substantial reduction when exposed to high Cu^{2+} concentration. Zheng *et al.* (2004) worked on the response of three ornamental crops (*Dendranthema grandiflorum* L. 'Fina', *Rosa hybrid* L. 'Lavlinger', *Pelargonium hortorun* L. 'Evening glow') to different solution levels of Cu^{2+} (ranging from 0.4-40 µM). They observed that excessive copper reduced the shoot and root dry weight of all three species. Manivasagaperumal *et al.* (2011) reported the decline of dry weight of *Vigna radiata with* increasing copper concentration of 100-200 mg/kg. According to Azooz *et al.* (2012), copper concentration above 10 mM reduced the dry weight of wheat.

From these observations it can be concluded that excess copper had some effect on germination, growth and dry matter yield of three commonly grown tea varieties. Varietal differences in response towards Cu^{2+} were observed in the shoot elongation study. However, in the roots, the differences was less prominent possibly due to the fact that Cu^{2+} accumulation occurs much more in the roots than in the shoots leading to a greater damage in the roots which minimizes the differential effect within varieties. Differences among cultivars in response to Cu^{2+} stress have been found in other plants such as *Triticum durum* (Ciscato *et al.* 1997), *Holcus lanatus* (Hartley-Whitaker *et al.* 2001) and *Kummerowia stipulacea* (Xiong *et al.* 2008). Inhibitory action of excess copper in root and shoot elongation and in their damage observed during the present study may be due to reduction in cell division, toxic effect on respiration and protein synthesis (Manivasagaperumal *et al.* 2011, Kupper *et al.* 1996 and Sonmez *et al.* 2006). Our results indicate that a detail study on the effect of excess copper on tea plants is warranted.

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Cultivation and yield of *Pleurotus sajor-caju* on various lignocellulosic substrates

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Abstract
Oyster mushrooms have ability to grow utilizing various lignocellulose substrates (Khan and Garcha, 1984). In our present work a comparative study on the cultivation and yield of *Pleurotus sajor-caju* using different compost composition were carried out. Six different lignocellulose materials like paddy straw, saw dust, shredded wood, rice husk, paper waste and sugarcane bagasse were used to prepare ten types of compost combinations for the cultivation of oyster mushroom. The shortest period for pinhead formation was determined to be 9 days on sugarcane bagasse compost, where as the longest period for the same was determined to be 18 days on paddy straw and saw dust combination. The total harvest period of oyster mushroom was noted to be 41 days for sugarcane bagasse and the longest harvest period noted was 55 days on paddy straw and saw dust mixture. The highest yield was 154.8 gm on paddy straw and sugarcane bagasse mixture (1:1).

Keywords: *Pleurotus sajor-caju,* compost, lignocellulose.

Introduction

Cultivation of oyster mushroom has been universally recognized for its nutritional value and minimal technology requirement. Cultivation of edible mushroom is a biotechnological process, which aids in reducing and equally protecting the environment from excess solid waste (Mshandete and Cuff, 2008; Sanchez, 2010). Oyster mushrooms have ability to grow at wide range of temperatures and utilizing various lignocellulose substrates. Importance of mushrooms as food and its medicinal value is now well recognized and hence, development of high commercial interest is noted worldwide. For this reason, there is need to have a constant supply of the substrates which is readily available with low cost price rather than depending on specific types of materials or some seasonal forest supply for cultivation of mushroom (Onuoha et al, 2009).

Several reports regarding the suitability of various substrates for mushroom production namely straws of rice (*Oryza sativa*), wheat (*Triticum aestivum*), ragi (*Elucine coracana)*, bazra (*Pennisetum typhoides*), sorghum (*Sorghum vulgare*), maize (*Zea mays*) (Bano et al., 1987; Goswami et al., 1987; Gupta and Langer, 1988) have been recorded. Utilization of woods of poplar (*Populus robusta*), oak (*Quercus leucotrichopora*), cotton stalk, pea shells and poplar saw dust (Philippoussis et al., 2001; Zervakis et al., 2001) has also been studied. Utilization of agricultural waste as a substrate for the production of mushroom plays a dual role in reducing the waste and at the same time being used as a fertilizer (Sher et al., 2011). The palm oil related study carried out by Pathmashini et al. (2008), Saidu et al. (2011) who reported sorghum, kurakkan, maize and paddy are suitable substrate for edible mushroom production. Many studies reported that organic supplement enhances the yield of mushroom, but Mane et al. (2007) corroborated that supplementation also increases the production, though not popular as a commercial ingredient for large scale production. The aim of this study is to investigate the cultivation of oyster mushroom (*Pleurotus sajor-caju*) on different substrates.

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Materials and Methods

Mushroom strain:

The strain of *Pleurotus sajor-caju* was collected from Mushroom Cultivation Unit of Ramkrishna Mission Ashrama, Narendrapur, Kolkata and was

maintained on Potato Dextrose Agar (pH 6.5), containing 40% potato extract, 2% dextrose and 2% agar.

Substrate Preparation and Cultivation:

Six different lignocellulosic materials like paddy straw, saw dust, shredded wood, rice husk, paper waste, sugarcane bagasse were used to prepare ten types of substrate composition for the cultivation of oyster mushroom. Mushroom grown on substrate using only paddy straw was taken as control set.

The substrates were separately soaked in water overnight and after 15 hours the materials were squeezed to drain off the excess water. Then the substrates were spread on the surface of clean blotting paper and air dried for 15 minutes. Wet substrates (500 gms) which was either individual lignocellulosic waste or combination of each lignocellulosic waste and paddy straw (1:1) ratio was taken for use as substrate for mushroom cultivation. Spawning was done in layering technique with 4-5 layers for each bed using 20% spawn (wet wt/ wet wt) in a nylon net bag. The spawned substrates were then put into 28 x 40 cm polythene bags, tightly closed and pin holes were made on the surface of the bag. Those bags were then kept in a hanging condition in the mushroom house for spawn running at 25 ± 1°C under dark condition until primordial was formed. After appearance of primordia (pinheads), the polythene bag was removed to allow normal development of fruit bodies at 22 ± 1°C and 80-90% relative humidity. Proper ventilation was provided to prevent increase of CO_2 concentration. Mushrooms were harvested in clusters manually.

The days required for spawn running, pinhead formation and fruitbody formation was recorded. The yield of mushroom (gm fresh weight) was noted upto three flushes in each case.

Results and Discussion

Spawn running- According to the results obtained from above experiment it is found that completion of spawn running took 12-22 days after inoculation of the substrate. All substrates were inoculated at the same day. The shortest spawn running period on sugarcane bagasse taking 12 days [Table 1, Fig: A]. These results are in agreement with Kulshreshtha et al. (2010) who reported nearly similar spawn running time for *Pleurotus florida* using various biological waste materials.

Pinheads formation- The pinheads formation is the next stage of mycelial growth during cultivation process. Small pinheads like structures were

Table 1: Days for completion of spawn running, pinheads formation and fruiting bodies formation using different substrates.

Name of Substrate	Days for completion of spawn running	Days for pinhead formation	Days for fruiting bodies formation (flush)		
			1st	2nd	3rd
Paddy Straw	17	22	26	33	43
Paper	17	25	29	39	50
Paper + Paddy straw	16	24	30	41	51
Rice husk	22	28	33	42	55
Rice Husk + Paddy Straw	19	24	27	36	48
Sawdust	18	25	29	40	54
Sawdust + Paddy Straw	21	28	32	41	52
Shredded Wood	19	26	32	42	54
Shredded Wood + Paddy Straw	17	23	28	37	49
Sugarcane Bagasse	15	20	24	36	50
Sugarcane Bagasse + Paddy Straw	12	16	22	33	48

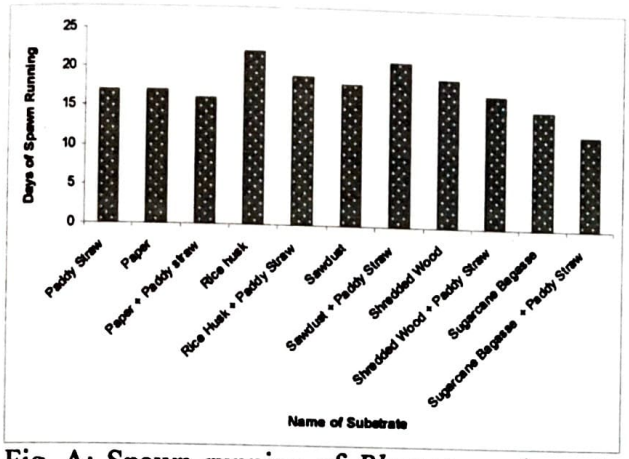

Fig. A: Spawn running of *Pleurotus sajor-caju* using different substrates

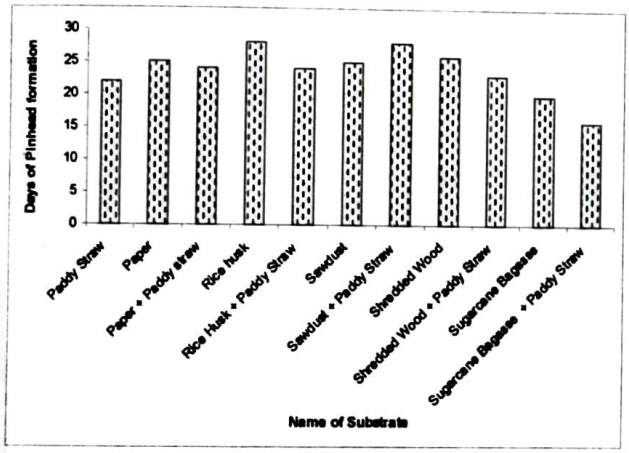

Fig. B: Pinhead formation of Pleurotus sajor-caju using different substrates

observed, these pinheads were formed 16-28 days after spawn running [Table 1, Fig: B]. these results agree with the findings of Shah et al. (2004) who studied the cultivation of oyster mushroom on different substrates.

Fruitbody formation- This is the final stage during the cultivation of mushroom. The fruiting bodies appeared 4-6 days after pinheads formation which is in conformity with Kulshreshtha et al. (2010) and took 8-13 days later after inoculation of spawn. [Table 1, Fig: C, Plate A].

Yield of oyster mushroom- The crop of oyster mushroom was harvested in three flushes. The maximum yield was obtained in first flush than the second and third flush. Maximum average yield of 154.8 gms was obtained from paddy straw and sugarcane bagasse mixture in (1:1) ratio (w/w). This is 121% greater yield than the control set (paddy straw, where the total average yield was

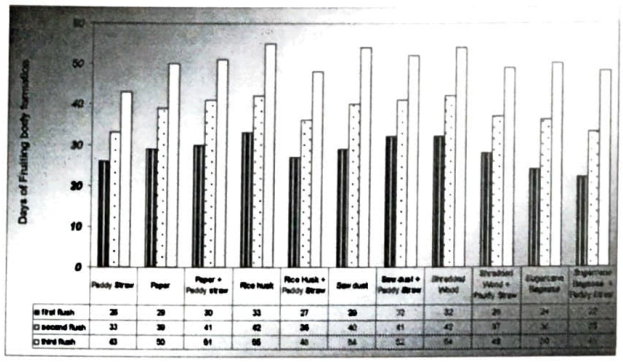

Fig. C: Fruitbody formation of *Pleurotus sajor-caju* using different substrates

Plate. A: Fruitbody formation of oyster mushroom

noted to be 127.9 gms only. This was followed by paper waste and paddy straw mixture and the minimum yield of mushroom were found to be 43 gm [33.62% of the control set] when shredded wood alone was used as the compost. [Fig: D, E]. It is reported that washed fresh sugarcane bagasse is viable as a substrate for the production of the

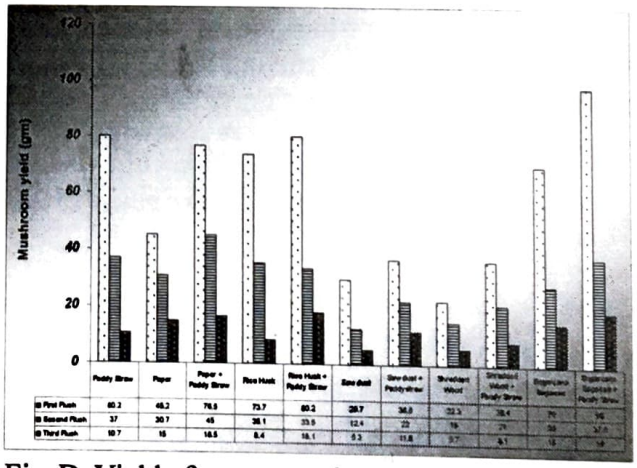

Fig. D: Yield of oyster mushroom (*Pleurotus sajor-caju*) in different substrates

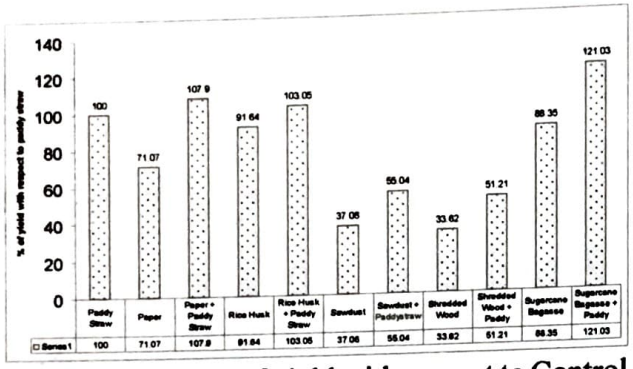

Fig. E: Percentage of yield with respect to Control set (Paddy Straw)

mushroom *Pleurotus sajor-caju*, especially in view of its low contamination and of a reduction in substrate disinfection cost [Moda et al. 2005]

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Footprint of Nitric oxide in induced systemic resistance

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Abstract
Nitric oxide (NO) is a potent signaling molecule with diverse physiological functions in plants. Several rhizobacterial strains may have capacity to induce systemic resistance in (ISR) plants but how far the biochemical mechanisms in which NO participates in this signaling pathway is still an open question. The present study have shown in *Pseudomonas aeruginosa* WS-1 mediated ISR inducing system in *Catharanthus roseus* induces defense enzyme and phenolics and also showed a two fold increase in NO production when challenge with *Alternaria alternata*. Furthermore, NO donor treatment in the host produced same defense molecules in a comparable manner. From those observations it is suggested that NO might have possible signaling role in ISR during crosstalk between the ISR inducing agent and pathogen within the host system.

Key words: *Alternaria alternata*, *Catharanthus roseus*, defense enzymes, Phenolics, *Pseudomonas aeruginosa*.

Other than innate resistance, plants could protect themselves from pathogens through acquired resistance which could be divided into two main categories i.e. systemic acquired resistance (SAR) and induced systemic resistance (ISR). Plants establish SAR when necrotic pathogen tries for an incompatible reaction with the host which is R gene mediated (Van Loon *et al.*, 1998) and ISR is achieved by the host when avirulent pathogen, elicitor molecules, different biocontrol agents, nonpathogenic plant growth promoting rhizobacteria (PGPR) interact with them (Pieterse *et al.*,1996; Van Loon, 2007; Pieterse *et al.*,2007). ISR is important because once induced in plants, may remain stable for a considerable part of their life time (Van Loon *et al.*, 1998). Both ISR and SAR represent a state of enhanced basal resistance of the plant which depends on different signaling molecules (Van Loon, 2007). Elucidation of signaling pathways controlling disease resistance is a major objective in research on plant-pathogen interactions (Pieterse *et al.*, 2007). There are several hypotheses in the signaling pathways of plant defense (Klessig *et al.*, 2000; Nandi *et al.*, 2003; Besson-Bard *et al.*, 2008). Recently, Nitric oxide (NO) has been emerging as a signaling molecule in plant disease resistance (Besson-Bard *et al.*, 2008; Acharya *et al.*, 2005; Acharya and Acharya, 2007; Hong *et al.*, 2008; Acharya *et al.*, 2011a, 2011b). Induction of disease resistance by production of enhanced level of defense enzymes has been reported in several plants to provide protection against invasion of pathogen attack (Friendrich *et al.*,1996; Van Loon *et al.*, 1998). Among the defense molecules, pathogenesis related (PR) protein like peroxidase (PO) plays a key role in biosynthesis of lignin to limit the extent of pathogen spread and also a component of early response in pathogen attack (Bruce and West, 1989), Polyphenol oxidase (PPO) could produce antimicrobial compounds and lignin, while, phenylalanine ammonia lyase (PAL), is a key enzyme in the phenylpropanoid pathway could perform defense related functions (Wen *et al.*, 2005). On the other hand, phenolic compounds are considered to be an important component of the disease defense mechanism (Nicholson and Hammerschmidt, 1992). During early interaction between ISR inducing bacteria and the host, the bacteria must produce one or more signaling compound as they are spatially separated from the inducing agent and giving systemic protection even against foliar pathogens (Kloepper *et al.*, 2004).

The present investigation was undertaken to evaluate the role of NO in the induction of ISR taking *Catharanthus roseus* as a model plant,

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Pseudomonas aeruginosa WS-1 as ISR inducing agent and *Alternaria alternata* as pathogen, based on the production of different defense molecules as mentioned earlier.

Materials and Methods

Strains

The pathogen, *Alternaria alternata* was isolated from infected *Catharanthus roseus* leaves with typical blight symptoms (Maity *et al.*, 2007). The fungal pathogen was grown on potato dextrose agar (PDA, Himedia, Mumbai, India) medium at 30°C. The biocontrol *P. aeruginosa* WS-1 was obtained from our laboratory culture stalk. The antagonist has subcultured and maintained on triptic soy agar (TSA, Himedia, Mumbai, India) medium for subsequent use.

Treatment

P. aeruginosa WS-1 was used in the induction of defense reaction in *C. roseus* plants. *C. roseus* (two months old) plants were grown in pots (each pot contain one plant) containing sterile soil and maintained at 28±2°C, in the green house. For each pot 100 ml of bacterial suspension at a concentration of 3×10^6 cfu/ml was used to drench the soil. One day after bacterization, one set of bacterized plants were challenged inoculated with spraying of 20 ml of *A. alternata* at a concentration of 3×10^5 cfu/ml / plant (Set- P.a.+A.a.) and another set of bacterized plants was not challenged with pathogen (Set- P.a.). Plants without prior treatment of bacteria were inoculated with pathogen at the same cfu (Set- A.a.). Plants neither treated with bacterial suspension nor challenged by pathogen were kept as control (Set- Control). The humidity of the green house was maintained at around RH 85%.

Enzyme extraction

The fully mature leaf tissues collected from different treated sets after treatment for successive day, were homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant to be used for the enzymatic assay was transferred to a 2 ml vial and stored at -80°C. The standard Bradford assay (1976) was employed, using bovine serum albumin as a standard, to test the protein concentration of each extract.

Peroxidase assay (PO)

Peroxidase activity was assayed spectrophotometrically following the method of Hammerschmidt *et al.* (1982). The reaction mixture consisted of 1.5 ml of pyrogallol, 0.5 ml of enzyme extract 0.5 ml of 1% hydrogen peroxide. The change in absorbance at 420 nm were recorded at each 30 sec intervals for 3 min. the enzyme activity was expressed as changes of absorbance of reaction mixture min^{-1} g^{-1} protein.

Phenylalanine ammonia lyase assay (PAL)

PAL was assayed following the method of Dickerson *et al.* (1984) determining the conversion of L-phenylalanine to transcinnamic acid spectrophotometrically at 290 nm. 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1M borate buffer (pH 8) and 12 mM L-phenylalanine in the in the same buffer for 30 min at 30°C. Enzyme activity was expressed as synthesis of transcinnamic acid (n mol) min^{-1} g^{-1} protein.

Polyphenol oxidase assay (PPO)

PPO activity was determined according to the method of Mayer *et al.* (1965). 200µl of 0.01M catechol was added to the reaction mixture containing 200 µl of enzyme extract and 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5). Enzyme activity was expressed as change in absorbance at 495 nm min^{-1} g^{-1} protein.

Phenol estimation

Leaf samples (1 g) as mentioned earlier were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C (Ziestin and Ben-Zaken, 1993). One ml of the methanolic extract was added to 5 ml of distilled water and 0.250 ml of 1 N Folin-Ciocalteau reagent and the solution was kept at 25°C. Phenolic content was measured spectrophotometrically at 725 nm using catechol as standard. The amount of phenolics was expressed as µg catechol /g protein.

Nitric oxide estimation

Production of NO was estimated by hemoglobin

assay (Acharya and Acharya, 2007; Hong *et al.*, 2008). 100 mg of leaf tissue was incubated in a reaction mixture containing 10μM L-arginine, 30μM hemoglobin, in a total volume of 2.0 ml of 0.1M phosphate buffer (pH 7.4) at 37°C. Production of NO was measured by using scanning spectrophotometer Hitachi 330 at 575nm as picomolar of NO produced per mg of protein per hour.

Real time NO production was visualized using membrane permeant fluorochrome 4-5-diammino-fluorescein diacetate (DAF-2DA) dye (Bartha *et al.*, 2005). Lower epidermis of leaf was peeled off and placed in a brown bottle containing 1 ml of loading buffer 10 mM KCl, 10 mM Tris HCl (pH 7.2) with DAF-2DA at a final concentration of 10μM for 20 min in dark. Fluorescence was observed with Leica DMLS microscope at excitation wavelength 480nm and emission wavelength 500-600 nm.

Treatment with SNP

Plants were treated with popular NO donor, 100 μM sodium nitroprusside (SNP) by foliar spray and exposed to normal day light. After 24h of treatment mature leaves were harvested, washed thoroughly and utilized for assay of PO, PPO, PAL and phenolics.

Results and discussion

ISR (or) SAR mechanism produces response to local attack by producing defense related compounds thereby reducing or inhibiting further attack by herbivore or pathogens (Hunt *et al.*, 1996; Sticher *et al.*, 1997; Hammer Schimidt, 1999).

Plants have various defense related genes but those are sleeping genes and appropriate stimuli or signals are needed to activate them by prior application of biological inducer which is thought to be a novel plant protection strategy (Radjacommare *et al.*, 2004). In this regard some of these biological control strains like Plant Growth Promoting Rhizobacteria (PGPR) mediated ISR against a broad spectrum of pathogens is being considered as most desirable approach in crop protection (Sticher *et al.*, 1997; Anand *et al.*, 2009). However, the molecular basis of signaling mechanism regarding the development of ISR induced broad spectrum protection is still an open area for research. In the present study defense enzymes systemically induced in host plant by *P. aeruginosa* WS-1 treatment significantly began from 24 h after challenge inoculation, the activity increase further and reached considerably higher on 4th day after treatment. Higher levels of induction of enzymes i.e. PO, PPO, and PAL, were observed in P.a.+A.a. set of *C. roseus* treatment (Fig. 1). Higher accumulation of total phenolics was observed in P.a.+A.a. treated set (Fig. 1). An increased activity of defense enzymes with higher total phenol levels like 26%, 44%, 56% and 23% of PO, PPO, PAL and phenolics respectively over the control were showed in case of P.a.+A.a. treated set after 4th day of incubation. Whereas no marked changes were observed in defense enzymes and total phenolics in untreated (control) as well as *P. aeruginosa* treated plants (P.a. set) which had not been challenged inoculated by the pathogen. In the pathogen treated set only a slight deviation of

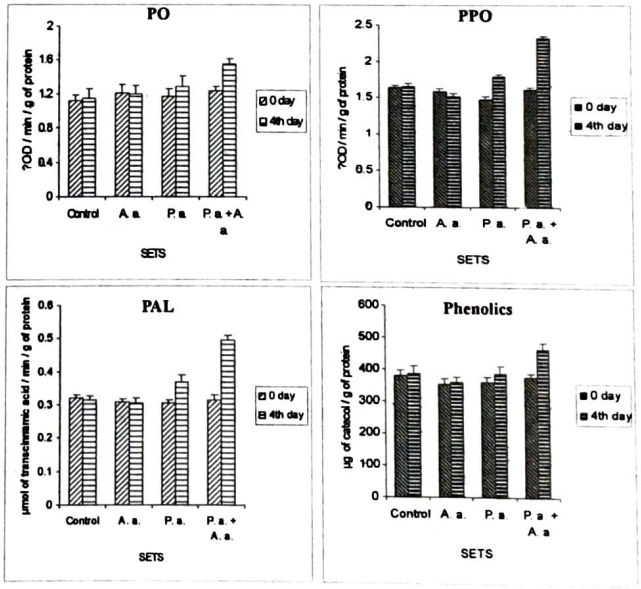

Fig. 1. Effect of application of biocontrol agent *P. aruginosa* WS-1 on the production of PO, PPO, PAL and phenolics on *C. roseus* leaf. The level of enzymes and phenolics production measured on the 4th day of treatment. Values represent mean ± SD of three separate experiments, each in triplicate. Control; A.a.- treatment with *A. alternata*; P.a.- treatment with *P. aeruginosa* WS-1; P.a. + A.a.- treatment with *P. aeruginosa* followed by *A. alternata*.

defense enzyme activity was observed. According to Sendhil vel (2003) in grapevine plants pretreated with *P. fluorescens*, and Anand *et al.* (2009) in chilli plant pretreated with *Colletotrichum capsici* did not show enhancement of defense molecule production without challenged with respective pathogen. In our case also it is interesting to note that sole application of *P. aeruginosa* WS-1 in the rhizosphere did not insist production of these defense weapons significantly, but enhance the production only when the plant challenged with the pathogen.

A two fold increase in NO production was observed in the ISR inducing system (set- P.a + A.a) on the 4th day after treatment (Fig. 2 Inset). It was further proved by real time NO visualization by using DAF-2DA, a fluorophore widely used for the detection and imaging of NO. DAF- 2DA provide the advantages of sensitivity, specificity and noncytotoxic and permitted the detection of intracellular NO (Lamotte *et al.*, 2004). Fig. 2 shows that ISR system induces NO production

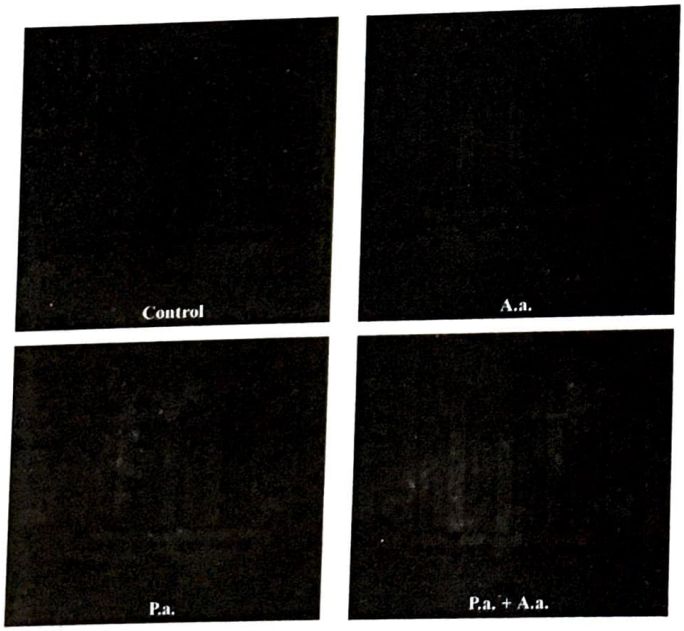

Fig. 2. Nitric oxide visualization in *C. roseus* epidermal cells by DAF 2DA, on the 4th day after treatment of different agents. NO generation is detected by green fluorescence. Inset: Production of nitric oxide by different set. Values represent mean ± SD of three separate experiments, each in triplicate. Control; A.a.- treatment with *A. alternata*; P.a.- treatment with *P. aeruginosa* WS-1; P.a. + A.a.- treatment with *P. aeruginosa* followed by *A. alternata*. Bar=10 µm.

which was further confirmed by realtime NO generation by DAF- 2DA.

Thus, from the observations it could be speculated that the chemical crosstalk between the ISR inducing agents and the pathogen might inflame the plant to produce elevated level of NO in the system. This observation insisted to investigate whether NO is the candidate taking part in the transduction of the message to produce defense weapons systematically. According to Gauples *et al.* (2008) NO act as a transducer of stress signal in plant system. So, to check the role of NO, we treated plants directly with NO donor SNP and they showed higher production of all those defense molecules (Fig. 3) which suggest that rhizobacteria mediated ISR might be governed by NO but how the signal is perceived and translocate by the plant is still to be investigated.

Our results suggest that activities of defense enzymes and accumulation of phenolics in the host induced by antagonist in response to challenge inoculation with the pathogen and also same system resulted in the induction of NO production. Furthermore, SNP treatment showed the elevation

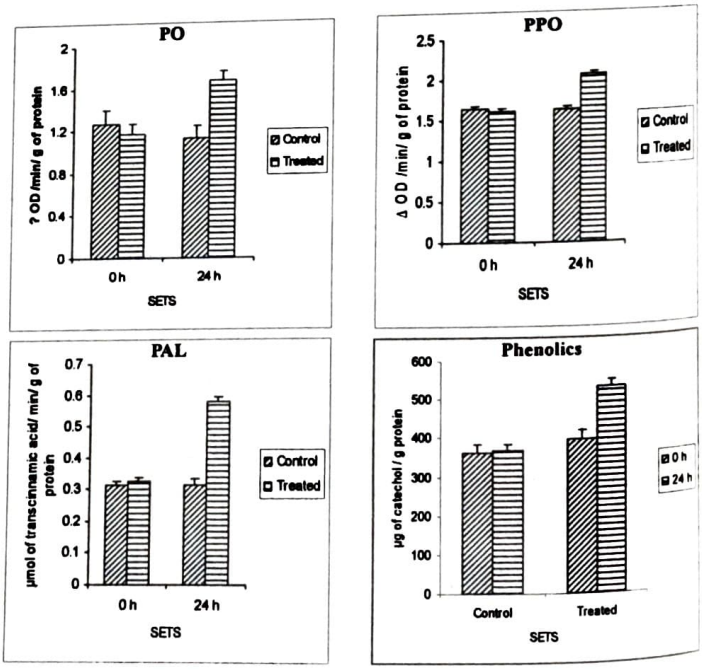

Fig. 3. Effect of SNP (100 µM) on the production of PO, PPO, PAL and phenolics after 24h of treatment. Values represent mean ± SD of three separate experiments, each in triplicate. Control; A.a.- treatment with *A. alternata*; P.a.- treatment with *P. aeruginosa* WS-1; P.a. + A.a.- treatment with *P. aeruginosa* followed by *A. alternata*.

in the production of same defense molecules over control. In mammals, NO circulate in the blood as either a S-nitroso protein adduct or as low molecular weight S-nitroso thiols such as nitroso glutathione (GSNO). This molecule believed to act as both an intra- and inter-cellular NO carrier, is a powerful inducer of plant defense gene (Durner *et al.*, 1998). Since glutathione is a major metabolite in the phloem (Maria *et al.*, 2003), where the ISR is transmitted, it can be hypothesized that excess NO produced during interaction binds to glutathione; in this form it may act as a long distance ISR signal. All the experimental data suggesting that during ISR, plants may be able to modulate the defense activity and signaling function of this stabilized form of NO.

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Genetic profiling of a small heterogeneous population presenting traditional, wild and wild relatives of rice (*Oryza sativa* L.) in relation to osmotic stress tolerance

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Abstract
Biochemical and genetic profiling of five rice lines which included one high yielding drought and salt sensitive cultivar (IR36), one drought avoiding indigenous line (Gorah), one salt tolerant popular cultivar (Nona Bokra), one wild rice (*Oryza rufipogon*) and one wild halophytic rice relative (*Porteresia coarctata*) were done in relation to osmotic stress tolerance. Biochemical analyses were done for three compounds (proline content, total chlorophyll and total protein) which are quantitatively vary under different osmotic stresses. The rice lines were genotyped for seven osmotic stress tolerance linked rice SSR loci and DNA sequence analysis was done for the amplified product of a salt inducible gene (*salT*) using two sets of allele mining primers. The varied biochemical profiles and growth habitat of the studied rice lines were partially confirmed both by the fingerprint analysis using rice SSRs (Simple Sequence Repeats) and DNA sequence analysis of the amplified product for the selected salt inducible gene.

Key words: Rice, Osmotic stress, *Saltol* QTL, SSR fingerprint, Microsatellite panel, *SalT* gene

Land races, wild species and wild relatives of different crop genera, growing in varied agro-ecological niche, constitute a rich source of crop genetic diversity. Investigation, cultivation and preservation of those play an important role in sustainable agriculture, resulting in conservation of crop genetic resources through inhibition of monoculture (Newton *et al.* 2010). Rice like other crops have a long list of such valuable hidden genetic resources that harbour a number of favourable genes that can be exploited in rice breeding programme for development of new cultivars (Ogbu *et al.* 2010). The first step for proper utilization and popularization of these germplasm in breeding programme is the proper characterization for the different desirable agronomic traits. As all the agronomically important traits are under polygenic control, the only way to dissect those traits is development of trait linked markers and respective QTL identification (Ram *et al.* 2007, Lang and Buu 2008). Once a trait linked marker is developed, it can be used for screening of a number of genotypes in relation to the target trait. In our earlier work (Karmakar *et al.* 2012) we have profiled a total of 10 typical drought tolerant traditional upland rice lines using trait linked molecular markers. The objective of the present work was proper biochemical screening, trait linked marker based genotyping and DNA sequence analysis for a candidate gene using two different sets of allele mining primers in relation to osmotic stress tolerance for a small heterogeneous collection of rice genotypes which included commonly grown cultivars, wild species and also wild relatives.

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Materials and Methods

Plant Materials

A total of five rice genotypes were investigated, the detailed descriptions including their growth habit, distinctive morpho-taxonomic identity, specific physiological notes and place of collection are in given in Table 1.

Biochemical screening

Quantitative estimation of three biochemical compounds (proline, total chlorophyll and total protein) commonly associated with vital life processes of plants were done from 30 days old

Table 1. Detail description of the studied five rice genotypes

Our Acc. number	Name of the Genotype	Growth/habitat	Distinctive Morpho-taxonomic character	Special physiological properties	Place of collection
VB9	IR 36 (*O. sativa* L. var. IR 36)	High yielding variety, developed in IRRI, now common in all rice growing region having irrigation facility.	Grain Golden Yellow in colour with Length and Breadth ratio (L/B) 3.83, kernel white with L/B ratio 3.35.	Highly osmotic stress (drought and salinity) susceptible.	Bose Institute farm, Kolkata, India.
VB17	Gorah (*O. sativa* L. var. Gorah)	Less cultivated indigenous line grown in drought prone area of Jharhkhand and Rarh Bengal.	Grain grey in colour with L/B ratio 2.53, kernel brownish with L/B ratio 2.25.	Typical drought avoiding line with short life cycle duration (about 70 days).	Nadia, West Bengal, India.
VB18	*O. rufipogon Griffiths* (Popular name Red rice and by some tribal as Orhidhan)	Wild rice species, annual but some plants grown in small shallow water body grows as perennial.	Grain yellowish redish in colour with L/B ratio 3.94 and kernel redish in colour with L/B ratio 3.72. Grains having a typical long awn.	Grown with rice as weed, shatter readily before harvest, so that field became thoroughly infested with dropped seeds, which can grow with the next crop, remain viable for 3 years.	Jamtora, Bankura, West Bengal, India.
VB170	Nona Bokra (*O. sativa* L. var. Nona Bokra)	Traditional popular cultivar, commonly cultivated in South India and also in coastal area of Sundarban, Bay of Bengal, used as donor in salt breeding programmes.	Grain Brownish yellow in colour with L/B ratio 2.88 and kernel light brownish with L/B ratio 2.86.	Typical salt tolerant lines and Extreme late heading.	Central Saline Soil Research Institute, Canning, 24-Pargana, West Bengal, India.
VB174	*Porteresia coarctata* (Roxb.) Tateoka (Syn. name *O. coarctata* Roxb.) (local name Bunodhan)	A tetraploid wild rice relative, grows as mangrove communities along the estuaries in India.	Leaf narrow with sharp edges and spiny tip, spikelet non-conventional, grain and kernel minute, Endosperm hardy, embryo typically large with epiblast, and scuteller tail.	Highly salt and submergence tolerant, can withstand up to 36% salinity, typical perennial grass.	Estuaries, Canning located at Bay of Bengal, 24-Pargana, West Bengal, India.

fresh leaf tissue using standard protocol (Roy *et al.* 2009, Sadasivam and Manickam 2010, Karmakar *et al.* 2012 respectively).

Genetic profiling using osmotic stress tolerance linked rice SSRs

Isolation of genomic DNA

Genomic DNA of the studied rice genotypes were isolated from fresh healthy seedlings, following a pre-standardized protocol (Lodha *et al.* 2011, Roychowdhury *et al.* 2012) of our laboratory and the concentration was adjusted to 25 ng/µl.

Marker selection for genotyping

Seven salt tolerance linked rice SSR loci [Rice Microsatellite (RM)] associated with *Saltol* QTL spanning 1.4 Mb positioned at 10.9 Mb to 12.3 Mb on rice chromosome 1 of *indica* rice (Bonila *et al.* 2002, Niones 2004, IRGSP 2005, Mohammadi-Nejad *et al.* 2008, 2010) and mapped between 11.1Mb to 14.6 Mb in a japonica rice (Haq *et al.* 2010, Thomson *et al.* 2010) were selected for genotyping of the studied lines. A freely available comparative web data resource of cereal crops (Gramene database, www.gramene.org) was used for collection of detailed information of these markers. The selected primers were synthesized from Integrated DNA Technologies (IDT, USA), the details of selected SSR markers are given in Table 2.

PCR amplification

PCR amplification was done in 25 µl of reaction mixture containing 4 µl of genomic DNA (100 ng), 2.5 µl of 10X Taq-buffer, 1.0 µl of 50 mM $MgCl_2$, 0.25 µl of 2.5 mM dNTPs, 1.0 µl of each of the

Table 2. Details of the used rice SSR and gene specific allele mining primers

SSR	Ann. Temp. (°C)	Repeat Motif	Nucleotide sequence	
			Forward Primer (5′-3′)	Reverse primer (5′-3′)
RM1287	55	$(AG)_{17}$	GGAAGCATCATGCAATAGCC	GGCCGTAGTTTTGCTACTGC
RM8094	51	$(AT)_{31}$	AAGTTTGTACACATCGTATACA	CGCGACCAG TACTACTACTA
RM3412	55	$(CT)_{17}$	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
RM10745	59	$(TATG)_9$	TGACGAATTGACACACCGAGTACG	ACTTCACCGTCGGCAACATGG
RM10764	58	$(AT)_{28}$	AGATGTCGCCTGATCTTGCATCG	GATCGACCAGGTTGCATTAACAGC
RM493	56	$(CTT)_9$	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
RM140	61	$(CT)_{12}$	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG
SalT gene (Z25811)				
Pr-5 (*SalT*5'-3')	55	--------	CCACGAAGACTATGACGCTGGTG	CTTTGACCACTGGGAATCAAGG
Pr-6 (*SalT* NC)	55	--------	ATGACGCTGGTGAAGATTGGCC	GGTGGACGTAGATGCCAATTGC

forward and reverse primer (at a concentration of 10 pmole/µl) and 0.1 µl (5 U/µl) Taq-DNA polymerase. The PCR profile starts with 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min with a final extension at 72°C for 7 min using a thermal cycler (M. J. Research, MC 013130). All the PCR reagents were purchased from Fermentas Life Sciences, USA.

Polymorphism screening, scoring and analysis of the amplified products

The amplified products were resolved through 6% native polyacrylamide gel and documented using a gel documentation system (Perkin Elmer, Geliance 200 imaging system). The molecular weight of the different amplified products (allelic variants) of the SSR markers across the studied lines was determined using analysis software (AlphaEaseFC 4.0, USA). The banding patterns obtained were scored in a binary matrix and used for cluster analysis considering the statistical parameter (complete linkage between and Euclidean distance among the groups) using the statistical software SPSS 10.0. For find out the polymorphism detecting ability of the used RM markers across the studied lines polymorphism information content (PIC) value of each marker was calculated following a formula ($PIC_i = 1 - \text{“}P^2_{ij}$ where i = 1 to n and P_{ij} represents the frequency of j^{th} allele for the i^{th} marker (Liu and Muse, 2005).

Allele mining for salt inducible tolerance gene

For allele mining of salt-inducible rice gene (*salT*, Z25811) two different sets of primers were used as described by Latha *et al.* (2004). The first one was the 5′-3′ primer selected from 5′ and 3′ untranslated region immediately outside the coding region, for amplifying the total coding portion along with introns. The second was the NC primer selected only from the coding region close to the regions encoding the N- and C-termini of the coded protein. The details of the primer sequences are given in Table 2. For PCR amplification same profile was followed as in SSR genotyping. The amplified products were resolved in 1.5% agarose gel for PCR conformation. The amplified products were purified using Quiagen gel extraction kit and sequenced from a sequencing company (Chromus Biotech, Bangalore, India) and the derived sequences were analyzed using bioinformatics tools.

Results and Discussion

Proline content (µg), chlorophyll content (mg) and total protein content (mg) quantified from fresh mature tissue for the studied rice lines are given in Table 3. The proline content estimated for the studied rice lines showed no correlation with their growth habitat, particularly to osmotic stress tolerance except for one genotype. Though IR36 is a high yielding variety and very much susceptible to drought and salinity, it showed the highest proline contents among the investigated lines. But being

Table 3. Overall biochemical estimation for the studied genotypes

Genotypes	Proline content (μg/g)	Chlorophyll content (mg/g)	Total Protein (mg/g)
VB9	72.8	0.889	36.9
VB17	61.2	1.753	41.4
VB18	43.6	0.791	79.3
VB170	52.4	1.690	55.4
VB174	58.8	1.629	48.5

highly salt tolerant, both the Nona Bokra and *P. coarctata*, showed lower proline content than IR36. Again Gorah, a drought avoiding line showed the proline content in between the IR36 and the later two genotypes. But for the rest genotypes, *O. rufipogon*, the lowest proline content can be correlated with its susceptibility to osmotic stress. Chlorophyll content was recorded to be highest in Gorah, a typical drought avoiding line, next to this are found in Nona Bokra and *P. coarctata*, both of which are typically salt tolerant line. The lowest chlorophyll content was found in IR36 and *O. rufipogon* which are not at all tolerant to any osmotic stresses. So this finding can be correlated with their stress reaction properties as well their growth behavior. Total protein content was recorded maximum for *O. rufipogon* followed by Nona Bokra, *P. coarctata*, Gorah and least in IR36. In this finding, except the first lines rest others are reflecting their growth habitat parameters. The fingerprint profile showed that two microsatellite markers (RM10764 and RM140) were monomorphic whereas rest five (RM1287, RM8094, RM3412, RM10745 and RM493) were polymorphic for the studied genotypes. Number of total alleles, amplified product size and PIC value of each SSR with the reported product size of the amplified product for reference genotype (*O. sativa* var. Nipponbare) are given in Table 4. A total of 18 different alleles were detected for the five polymorphic loci across the studied genotypes with maximum numbers (4) as recorded for

Table 4. Number of detected alleles, amplified product size, PIC value and product size in reference genotype (O *sativa* var. Nipponbare) for the studied SSR loci

SSRs	No. of alleles detected	Amplified product size (bp) range	PIC value	Product size (bp) for reference genotype
RM1287	4	150 – 226	0.72	162.00
RM8094	4	177 – 203	0.84	209.00
RM3412	4	166 – 227	0.72	211.00
RM10745	3	166 – 186	0.56	189.00
RM10764	Monomorphic	158	0.00	237.00
RM140	Monomorphic	267	0.00	261.00
RM493	3	179 – 237	0.56	211.00

Table 5. Microsatellite marker panel

Genotypes	RM 140	RM 10764	RM1287				RM8094				RM3412				RM10745			RM493		
	A	A	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	A	B	C
VB9	267	158	150						196			216					186		218	
VB17	267	158			185		178						227				186			237
VB18	267	158		170				177					227			176			218	
VB170	267	158	150							203				203			186		218	
VB174	267	158				226					166				166			179		

RM1287, RM8094 and RM3412 and minimum (3) for RM10745 and RM493. A microsatellite panel (Table 5) was constructed using the molecular weight (base pair) of different detected alleles resulted from SSR fingerprints. The amplified product size range was found to be broadest (150 bp – 226 bp) for RM1287 and narrowest (166 bp – 186 bp) for RM10745. The SSR fingerprint derived dendrogram (Fig 1) revealed that IR36 and Nona Bokra form one subcluster to which Gorah and *O. rufipogon* joined to form a cluster. To this cluster, *P. coarctata* joined individually to form the final complete cluster. The calculated PIC value for the polymorphic markers showed its range from 0.84 (for RM8094) to 0.56 (for RM493 and RM10745).

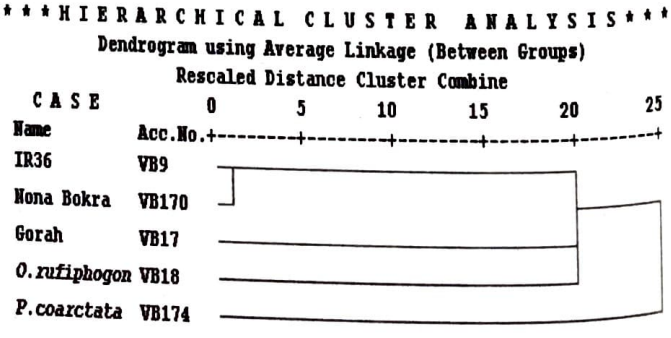

Fig 1. SSR fingerprint derived dendrogram

From the microsatellite panel it can be concluded that the five polymorphic SSR loci are highly diversified and showed about 3.6 alleles/ SSR loci across the studied five rice lines. The deviation in size of amplified product from the reference genotype (as described in Table 4) for the studied SSR loci are not so significant except for RM 10764 and RM 494. The reason for this deviation is the nature of reference genotype (Nipponbare) which is a *japonica* rice line. The PIC value for the polymorphic SSR loci is significantly high and above 0.50 which also indicating their high distinguishing ability for studied genotypes. In SSR fingerprint derived dendrogram the three true rice genotypes were grouped together to which *O. rufipogon,* the wild rice joined. *P. coarctata,* the wild rice genus became separated from the earlier cluster. Though this clustering pattern do not reflecting their growth behavior pattern, the taxonomic identity are reflected, as *O. rufipogon* is regarded as the immediate progenitor of present rice (*O. sativa*), whereas *P. coarctata* is considered as a distinct genus under the tribe *Oryzeae*. So, it can be concluded that the used SSR loci spanning the *Saltol* QTL is not sufficient for dissecting genetic diversity of this heterogeneous collection in relation to osmotic stress tolerance but have the ability to separate the studied genotypes based on their taxonomic identity. For getting more appropriate results, additional drought tolerance QTLs with linked markers should be included in addition to *Saltol* QTL. The DNA sequences obtained from the amplified products using two different primers of *salT* gene were bioinformatically analyzed and used for phylogram analysis using ClustalW2 programme which showed two different clustering patterns. The dendrogram constructed for Pr-5 (*salT* 5′-3′) (Fig 2) showed that *O. rufipogon* and

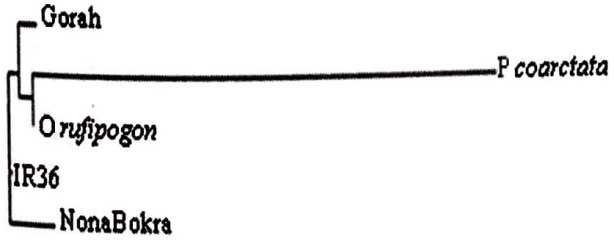

Fig. 2. DNA Sequence deried phylogram for *salT* (5'-3') primer

P. coarctata form a subcluster to which Gorah joined separately to form a cluster. The rest other genotypes (IR36, Nona Bokra) and this cluster joined separately to form the final cluster. Dendrogram derived from Pr-6 (*salT* NC) (Fig 3) showed that Gorah and Nona Bokra form one sub cluster, to which IR36 joined to form a cluster. To this cluster *P. coarctata* and *O. rufipogon* joined individually to form the final cluster. In *salT* gene sequence derived dendrogram for the Pr-5 (*salT* 5^1-3^1) primer though both *O. rufipogon* and *P. coarctata* took part in clustering, the genetic distance between the rice cluster and *P. coarctata* is much more than that of *O. rufipogon* which is again more directive to taxonomic identity than the physiological parameters. This dendrogram showed common clustering pattern with the SSR derived dendrogram.

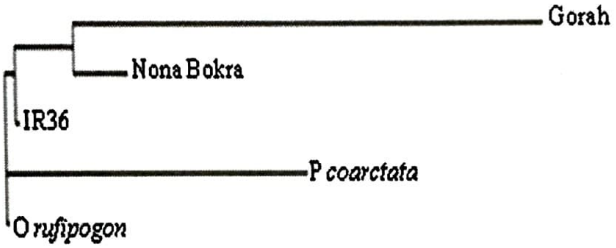

Fig. 3. DNA Sequence deried phylogram for *salT* (NC) primer

The inconclusive results derived from biochemical analysis may be due to use of normal tissue only. If stressed leaf tissues were considered in addition to the normal tissue, it may give a conclusive result. There are no earlier reports on these studied lines for the studied molecular markers spanning the *Saltol* QTLs in relation to osmotic stress tolerance and this may be the first one. The apparent lack of similarity among the clusters derived from different marker system may be due to consideration of less number of markers and respective QTLs. From sequence analysis it can be concluded that only one gene with its two sets of primers are not sufficient for sequence based profiling. Some additional osmotic stress induced genes (like *DREB, LEA*) should be considered for getting significant findings in addition to *salT* gene. Though this present study as a whole do not giving much more conclusive results, but it can be considered as a

preliminary study indicating the relationship among rice, wild rice and rice relatives in relation to salt tolerance.

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Screening of Zinc Resistant Bacteria Isolated from Coal Mine Overburden Soil

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Abstract
Heavy metal pollution of coal mine spoils is a significant environmental problem affecting both flora and fauna. These degraded soils can be remediated by using heavy metal resistant microorganisms. Zinc has been usually reported in high concentration from coal mine spoils. The present study, therefore, deals with screening of Zinc, resistant bacteria isolated from coal mine spoils. The bacterial isolates were isolated from coal mine spoils of Tinsukia district of Assam. For the isolation of bacteria, King's B Medium and Pikovskaya's Agar Media were used. Heavy metal resistance of the bacterial isolates against zinc was tested on nutrient agar and sucrose-minimal salt low phosphate (SLP) medium. The results showed that out of the 20 test isolates 11 isolates were resistant. The minimum inhibitory concentration (MIC) ranged from 0.001 M to 0.02 M. Antibiotic resistance was studied for two most resistant isolates. The optimal growth conditions with reference to pH and temperature of the two most resistant bacterial isolates were evaluated. Growth pattern of the most resistant isolates was determined in different concentrations of zinc amended broth media. On the basis of cultural and biochemical characters, the resistant isolates were identified as *Pseudomonas sp*, *Bacillus sp* and *Rhizobium sp.* Some of these isolates also exhibited plant growth promoting (PGP) traits and phosphate solubilizing ability.

Key words: Heavy metal; Resistance; Zinc; Minimum inhibitory concentration; PGP traits.

Open cast coal mining is one of the major factors leading to acidification and heavy metal contamination, which has poised a great threat to both biotic and abiotic factors (Kabata-Pendias, 1992; Jarup, 2003). Heavy metal contamination of soil is receiving increasing attention, particularly in developing countries (Yanez et al., 2002). The remediation of such soils is important because these usually cover large areas that are rendered unsuitable for agricultural and other human use. Moreover, elevated concentration of heavy metals can form complex toxic compounds and cause oxidative stresses, alter protein structure which severely influence biological functions (Rajbanshi, 2008). Many microorganisms can survive in harsh acidic and heavy metal contaminated soil as they can mitigate the toxic effects of heavy metals through secretion of acids, proteins, phytoantibiotics & other chemicals (Han et al., 2005). These indigenous heavy metal resistant microorganisms can play an important role in reclamation process (Ledin et al., 1996) as they are potent in biosorption, bioprecepitation, extracellular sequestration, transport mechanisms and chelation (Mergeay, 1991; Hughes and Poole, 1991). This study, therefore, aimed to isolate bacteria from coal mine overburden soil and to screen them with respect to zinc resistance and some growth promoting traits.

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Materials and Methods

Study area and sampling

The study area for the present work was Tirap colliery of Makum coal fields (latitudes 27°13'-27°23'N and longitudes 95°35'-96°00'E) of Tinsukia, district of Assam, India. The overburden soil was 15-20 yrs old, disorganized, poor in supportive and nutritive capacity. The rhizospheric soil samples of 12 different plants were collected during winter season 2010. Soil samples were aseptically collected using an auger from a depth of 10-15 cm according to V-shaped method (Bashan and Wolowelsky, 1987). Samples were taken to the laboratory in sterile plastic bags. The samples were then mixed in equal proportion to make a composite sample. The composite sample was sieved through a 4mm mesh sieve and was kept at field moisture

content at 4°C for until further analysis.

Soil physico- chemical analysis

Soil pH was determined using a digital pH meter (Cyberscan 510). Temperature of the overburden spoils were recorded by inserting a soil thermometer at a depth of 15 cm for 10 minutes. Moisture content (%) was calculated after drying 10 g of soil at 105°C for 24 hrs. Soil N was estimated by the alkaline permanganate method (Black et al., 1965). Available soil P was determined by the procedure of Jaiswal (2006) and available K was determined using flame photometer technique (Toth, S. J and A. L. Prince, 1949). The available Ca and Mg were determined by EDTA titration method (Tucker, B.B & L.T. Kurtz, 1961). Soil organic carbon was estimated by dichromate oxidation method (Walkley and Black, 1934).

Estimation of Zinc

Concentration of Zinc in the spoil was determined with the help of Atomic Absorption Spectrophotometer (Perkin Elmer AA200) by the triple acid mixture method (Jackson, 1967). Analytical conditions of atomic absorption spectrophotometer (AAS) used were as: wavelength - 213.86 nm, silt width - 2.7/1.80 mm, operating working range - 0.01 – 2 ppm, HC lamp current -5 mA, type of flame - Air– C_2H_2, fuel gas flow rate -1 L/min, air flow rate - 3.5 L/min.

Isolation of bacteria

Kings B media (HIMEDIA, Mumbai) and Pikovskaya's agar (HIMEDIA, Mumbai) media were used for the isolation of bacteria from the samples. Bacterial isolates were isolated by spread plate and streak plate method. Plates are incubated at 28°C for three days. All the pure culture isolates were maintained in the laboratory by subculturing on nutrient agar slant at an interval of 15 days.

Screening and Determination of minimum inhibitory concentration

(MIC) of zinc resistant bacteria

The isolated strains were screened for zinc ($ZnCl_2$) resistance by agar dilution method (Cervantes et al., 1986) using Nutrient agar and Sucrose-minimal salts low phosphate agar (SLP) media. Zinc chloride amended agar plates of various concentrations were inoculated with 0.5 ml of 1.0 O.D. actively growing cell suspension. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at 30°C for 24-48 h. The lowest concentration that prevented bacterial growth was considered as the minimum inhibitory concentration (MIC) (Summer and Silver, 1972; Aleem et al., 2003). Characterization of the resistant isolates was done using Bergey's Manual of Systematic Bacteriology (1994) and Microbiology A Laboratory Manual (Cappuccino & Sherman, 2006).

Antibiotic sensitivity of heavy metal resistant isolates

Antibiotic sensitivity and resistance of the isolated heavy metal resistant isolates were assayed on Mueller Hinton agar (HIMEDIA, Mumbai) plates according to the Kirby-Bauer disc diffusion method (Bauer et al., 1996). Plates were seeded with 0.5 ml of 1.0 O.D. actively growing cell suspension and allowed to dry. Antibiotic discs in triplicates were placed over the plates. After incubation at 30°C for 24-48 h, isolates were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone as described in standard antibiotic disc chart.

Growth studies

Growth pattern of the two most resistant isolates was studied in 0.1 M zinc chloride amended nutrient broth and un-amended control. Flasks were inoculated with 0.5ml of standard active inoculum and incubated in a rotary shaker (150 rev/ min) at 30°C. Growth was monitored as a function of biomass by measuring the absorbance at 600 nm using spectrophotometer (Systronics, Double beam, 2202).

Determination of optimal growth conditions

The optimal growth conditions of the two most resistant strains with reference to pH and temperature were determined. The isolates were grown in nutrient broth with range of pH values i.e. 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 and different incubation temperatures i.e. 25°C, 30°C and 37°C. The bacterial growth was determined by measuring the optical density of the log phase growing cultures at 600 nm.

Characterization of resistant rhizobacteria for PGP traits

Resistant bacterial isolates were also studied for their plant growth promoting traits. Production of indole acetic acid was (IAA) was detected as by the method of Brick et al., (1991). Ammonia and catalase were detected by the method as described by Cappuccino and Sherman (1992); phosphate solubilizing activity of the strains was determined by plate assay method (Pikovskaya, 1948).

Results

Physico-chemical characteristics of overburden spoils

Physico-chemical properties of coal mine spoils are presented in (Table-1). The soil was highly acidic and pH was found to be as low as 4.37. Soil temperature was 19.27°C having very low moisture in to it (4.56%). The spoil had high percentage of organic carbon (1.87%) but was poor in available P (43.68 Kg/ha. Total Nitrogen and available K were respectively recorded as 634.50 Kg/ha and 36.77 Kg/ha. Available Ca was 240.48 ppm while the available Mg 16.16 ppm. The concentration of zinc in the spoil was estimated to be 210.5 ppm.

Table 1 Physico-chemical characteristics of the rhizospheric overburden soil.

Physico-chemical Characteristics									
pH	Tem.	M (%)	C org. (%)	N total (Kg/ha)	Avail. P (Kg/ha)	Avail. K (Kg/ha)	Avail. Ca (ppm)	Avail. Mg (ppm)	Zn (ppm)
4.37	19.27	4.56	1.87	634.5	43.68	36.77	240.48	16.16	210.5

Table 2 Zinc sensitivity profile of the bacterial isolates.

Sl. No.	Isolates	0.5mM ZnCl2	Remarks
1	TP1	+	Resistant
2	TP2	+	Resistant
3	TP3	+	Resistant
4	TP4	+	Resistant
5	TP5	+	Resistant
6	TP6	+	Resistant
7	TP7	+	Resistant
8	TP8	-	Sensitive
9	TP9	+	Resistant
10	TP10	-	Sensitive
11	TP11	-	Sensitive
12	TP12	+	Resistant
13	TP13	+	Resistant
14	TP14	+	Resistant
15	TP15	-	Sensitive
16	TP16	-	Sensitive
17	TP17	-	Sensitive
18	TP18	-	Sensitive
19	TP19	-	Sensitive
20	TP20	-	Sensitive

Note: +, growth; -, no growth

Isolation, screening and characterization of resistant bacteria

Twenty bacterial isolates were isolated and screened for zinc resistance initially at 0.5 mM concentration. Out of the test isolates, eleven were found resistant, capable to grow on 0.5 mM zinc amended agar plates (Table-3). On the basis of cultural and biochemical traits, zinc resistant isolates belong to the genus *Bacillus*, *Pseudomonas* and *Rhizobium* (Table-3). Species level identification and molecular characterization are in progress. The minimum inhibitory concentration (MIC) of the resistant isolates was determined (Fig.-1). Two resistant isolates, *Pseudomonas* and *Bacillus* had highest MIC (20 mM $ZnCl_2$ conc.)

Antibiotic resistance, optimal growth and growth pattern

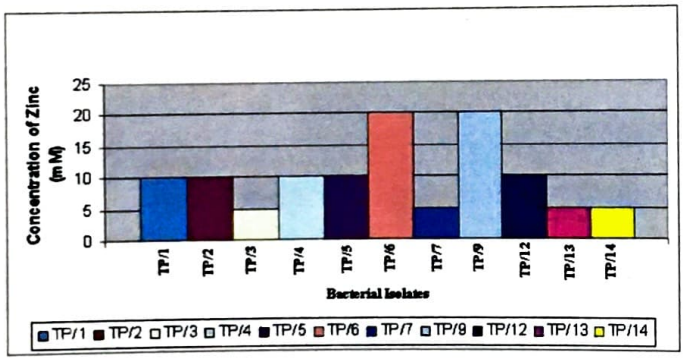
Antibiotic resistance, optimal growth and growth pattern of the two most zinc resistant isolates were evaluated. Strain TP6, characterized as *Pseudomonas* sp., and was found resistant to

Table 3 Morphological and biochemical characteristics of resistant bacterial strains.

Morphological and biochemical characteristics	*Bacillus* (5)*	*Pseudomonas* (5)*	*Rhizobium* (1)*
Gram reaction	G +ve	G -ve	G -ve
Shape	rods	rods	rods
Pigments	-	+	+
Dextrose	+	+	-
Sucrose	+	+	-
Mannitol	+	-	+
Oxidase	-	+	+
H2S production	-	+	+
Indole	-	-	+
Methyl red	-	-	+
Vogues Proskauer	+	-	+
Citrate utilization	+	+	-
Nitrate reduction	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	-	-

*Number within parenthesis shows no. of strains.

Fig.1- Effect of different concentrations of Zinc on the growth of Zinc resistant bacterial isolates.



tetracycline and ampicillin while, strain TP9 was resistant to ampicillin and streptomycin (Table-4). Optimal pH for growth of TP6 and TP9 strains was 6.5 and 7.0 respectively. The strains TP6 and TP9 required 28°C and 31°C respectively for optimum growth. Growth pattern of the strains TP6 and TP9 were studied both in control and 0.1 mM $ZnCl_2$ amended broth media (Fig. 3 and 4). The strains TP6 and TP9 respectively required 18 to 22 hrs and 22 to 24 hrs to reach the stationary phase. However, the growth of the two strains was slightly less in metal amended media than in control.

Plant growth promoting traits

All the resistant strains except strain TP3 (*Bacillus* sp) showed PGP traits (Table-5). Among the

Table 4 Antibiotic sensitivity profile of the two highest zinc resistant isolates.

Sl. No.	Isolate Code	Organism	Sensitive to	Resistant to
1	TP6	*Pseudomonas* sp.	Ciprofloxacin, Chloramphenicol, Streptomycin	Tetracycline, Ampicillin.
2	TP9	*Bacillus* sp.	Chloramphenicol, Ciprofloxacin, Tetracycline	Ampicillin, Streptomycin.

Fig. 3 Growth pattern of 20mM zinc resistant *Pseudomonas* sp.

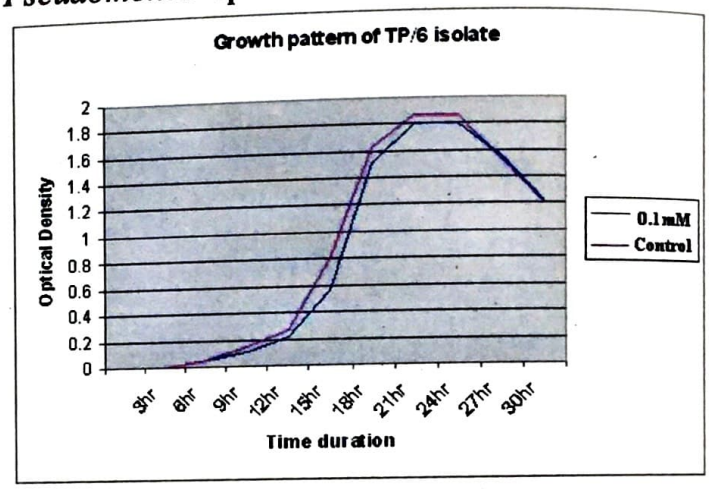


Fig. 4 Growth pattern of 20mM zinc resistant *Bacillus* sp.

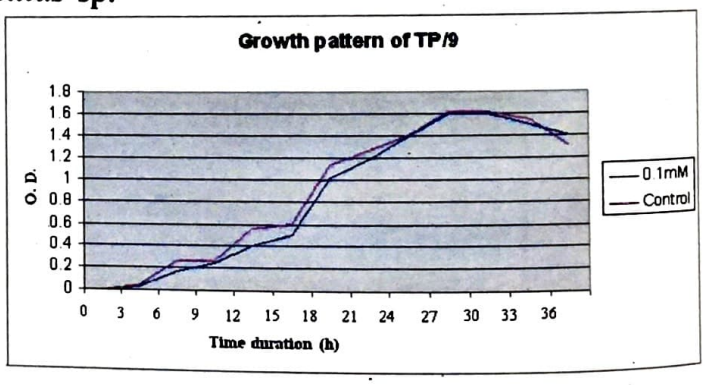


Table 5 Plant growth promoting traits (%) of the zinc resistant isolates.

Bacteria	No. of Isolates	*PGP characteristics (%)*		
		IAA production	Ammonia production	Catalase production
Pseudomonas spp.	5	100	100	100
Bacillus spp.	5	100	80	100
Rhizobium spp.	1	100	100	100

resistant isolates phosphate solubilizing activity was exhibited by strain TP5 (*Pseudomonas sp*), TP13 & 14 (*Bacillus sp*) and TP12 (*Rhizobium sp*).

Discussion

This study reveals that the coal mine overburden soil was acidic. All the soil physico-chemical parameters studied were more than optimum except the available phosphorus level that was low. This may be one of the critical factors limiting plant growth. Zinc concentration of the spoil soil was high. In the present investigation >50% of the isolates showed resistance to zinc. The isolates might have acquired varied mechanisms of zinc resistance ranging from reduced uptake to uptake and efflux, external and internal sequestration and in some cases transformation of metals to less toxic form due to zinc resistance genes and metalloregulatory proteins over a period of time. Presence of isolates having zinc resistance as well as phosphate solubilizing abilities might play an important role to increase the phosphate level of the phosphate deficient spoil soil. Acidity, zinc toxicity, nutrient availability and distorted soil texture may be the critical barriers on the way of revegetation of mine spoils. The indigenous resistant bacteria having plant growth promoting traits might have applications in the reclamation of degraded mine spoils. This needs to be explored properly so that appropriate ecofriendly techniques can be developed for the revegetation of degraded mine spoils.

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Ethno mycological Study of Wild Edible Mushrooms of Jammu and Kashmir

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Abstract
The state of Jammu and Kashmir has the largest concentration of forest dwellers, comprising of about one-fourth of the population of the state. Several tribes and villagers subsist largely on non-traditional and wild food sources especially wild edible mushrooms. Limited information, however, is known about ethno mycology of macrofungi in India, in general, and Jammu and Kashmir, in particular. Therefore, ethnomycological survey was carried out in various locations of Jammu and Kashmir state in order to develop a database on mushroom diversity and their traditional uses. In this study, forty four wild mushrooms which are actually consumed in the state and are distributed in twenty genera have been recorded to have various potentialities. These include *Agaricus* spp., *Boletus* spp., *Clavaria* sp., *Clavulina* spp., *Coprinus* spp., *Geopora* spp., *Gyromitra* spp., *Helvella* spp., *Macrolepiota* sp., *Morchella* spp., *Otidea leporina*, *Peziza* spp., *Pleurotus* spp., *Ramaria* spp., *Rhizopogon* spp., *Russula sp.*, *Sepultaria* spp., *Sparassis* spp., *Termitomyces* spp. and *Verpa* sp.. Their descriptive vocabulary and folk taxonomy, edibility status, traditional drying and preservation, culinary potential, common beliefs and traditions etc. have been highlighted in this paper. Overall, richness of ethno-mycological information gathered indicates that the Himalayan state of Jammu and Kashmir represents a mycophilic region where edible mushrooms play an important role in the socio-economic activities of the local populace.

Key words: Wild, Diversity, Mushrooms, Edible, Ethnomycology, Folk taxonomy

Mushrooms represent an imperative group of Kingdom Myceteae, which produce large fruiting bodies especially during the monsoon season from branching mycelium that infiltrates the soil, leaf litter, wood of living and dead trees. They have been commonly categorized into many groups like agarics, morels, truffles, boletes, puffballs, earthstars, stinkhorns and polypores.

Mushrooms are important to humankind and constitute the most relished food commodities amongst the number of non-conventional foodstuffs primarily because of their unique flavor and texture and are consumed either in fresh or processed form. They enjoy dietary supremacy over the rest of the vegetarian menu due to i) structural polysaccharides (glycogen, mannitol, trehalose etc.); ii) proteins comprising of nutritionally favourable proportion of essential amino acids thus capable of substituting meat; iii) negligible cholesterol content; iv) low lipid content; v) chitin contributing as a source of dietary fibre; vi) high vitamin B content; vii) Generally Recognized As Safe (GRAS) status by the U.S. Food and Drug Administration (FDA). Wild edible mushrooms are an important food source for rural communities during the rainy and winter seasons in many regions of the world. Mushrooms sold in regional markets in various parts all over the globe provide additional income to households (Hobbs, 1995; Rojas and Mansur, 1995; Boa, 2004; FAO, 2004; Sagar, et al., 2005).

The study of fungi in folklore, fiction and rituals from prehistoric times to the modern era is referred to as ethnomycology. It includes the indigenous knowledge, folk taxonomy and valuation of fungi. The field of ethnomycology only began to be realized in the1950's or 60's (Moerman, 2008). Archaeological evidences reveal edible mushroom species associated with people living 13000 years ago in Chile but it is in China where the eating of wild fungi was first reliably noted several hundred years before birth the of Christ (FAO, 2004).

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Worldover two types of societies namely, mycophilic and mycophobic, emerged over the period of time. The former adopted by many cultures especially in the Orient (throughout Asia, most of Europe including France, Italy, Poland, Romania, Spain), identified that certain mushrooms could have profound health-promoting benefits while in the latter existed a frightening fear of mushrooms which could even approach phobic levels (United Kingdom, Ireland and North America).

India, being a country of varied agro-climatic conditions, supports the existence of diversity of mushrooms in wild. Numerous species of wild growing mushrooms (macrofungi) are recognized and widely consumed as a delicacy across various regions of India since 3000 B.C. The earliest word in Sanskrit for mushrooms is 'Ksumpa' and Khumb' in Hindi while the word 'Chatra' is given to the fleshy-capped fungi.

Several ethnomycological studies have been carried out in various parts of India, (Pandey, and Singh, 1978; Harsh, *et al.*, 1993, 1996; Rai, *et al.*, 1993; Boruah, 1997; Kamat, 1999; Deshmukh, 2004). The local names of mushrooms vary from place to place. *Morchella* species in Himachal Pradesh, Jammu and Kashmir (J&K) and Uttarakhand are referred to as Guchchi'. *Agaricus* species in Uttarakhand as 'Cheun' and 'Khumbi' in Punjab and 'Khumb' in J&K. In Goa, people are mycophagic and consume local wild mushrooms commonly called as 'Olmi'. The forest dwelling backward community of Goa- the Velips, consumes *Russula* species. The species of boletes are known as 'Bhuifod' (Earth-boil) or 'Fuge' (Baloon mushroom). The termitophillic species abound in Goa are locally known as 'Roen Olmi' (termite hill mushroom) and their habitat is also venerated as the abode of the most popular goddess of Goa- Santeri. (Kamat, 1999). At least 50 delicious recipes from wild edible mushrooms are cooked in Goa (Kamat, 1999).

The state of Jammu and Kashmir, which lies in the north-west Himalaya, is stretched between 32°17'-37°03' N latitude and 72°03'-80°20' E longitude, and covers a total area of 2,22,235 sq.km., with an average annual rainfall between 60-80cm. It is bordered to the north and east by the main Himalayan ranges and Punjab plains to the south. The state exhibits varied climatic and topographic conditions and provide pleasant environment for the lavish growth of the dense coniferous and mixed forest of *Cedrus deodara* (Roxb.) G. Don, *Pinus wallichiana* A.B.Jackson, *Picea smithiana* (Wallich.)Boiss. *Abies pindrow* Royle, *Quercus* sp. L., *Juglans regia* L., *Alnus nepalensis* D.Don, *Ulmus wallichiana* Planch. etc. These forests encompass a rich repository of the macrofungal wealth that more or less remain unexplored and presently a little information on the diversity and ethno mycology of wild mushrooms has been highlighted from this northern most state of India (Kaul and Kachroo, 1974; Kaul, 1981; Kumar and Sharma, 2010). Therefore, we carried out an ethnomycological study in various locations of Jammu Province of Jammu and Kashmir state during the period 2005-2010, in order to develop a database on mushroom diversity and ethno mycological aspects.

Materials and Methods

Standard methods of collection, preservation, macro and microscopic studies were followed (Christensen, 1981; Corner, 1972; Purkayastha and Chandra, 1985; Kaul, 1997; Pegler, 1977; Soothill and Fairhurst, 1978; Smith *et.al.*, 1981; Arora, 1986; Singer, 1986; Kumar *et. al.*, 1990; Atri *et al.*, 2003) and the shape, size, and colour of fresh specimens were recorded before preservation. Ethnomycological information was recorded from reliable sources such as hakims, tribals, and local inhabitants who were considered to have good knowledge of the wild resources of the region. They were taken to the forests as guide cum informants. In order to gain better understanding of the relationship between the fungi, the local people, and the economy, field investigations and interviews were conducted in different local languages *viz.*, Bhadarwahi, Kishtwari, Gadaishi, Dogri and Kashmiri. The interviews were semi-structured having a set of questions which were put forth to the selected locals to ascertain their views on historical background, traditional usage, edibility

status, folk taxonomy, methods of drying and preservation, commercial importance etc. of fleshy fungi. Repeated interviews were conducted to substantiate and authenticate the information. The photography was accomplished using digital camera (Sony DSC-P92). Each specimen was collected and labeled, indicating number, date of collection, locality and uses. All collections have been deposited in the herbarium of Botany Department, University of Jammu, Jammu with accession numbers.

Results and Discussion

While carrying out ethnomycological studies in different localities of the study area, the local inhabitants were randomly interviewed in their native languages (Bhadarwahi, Kishtwari, Gaddaishi, Dogri and Kashmiri) and the analysis of the total use pattern of the wild edible mushrooms, preservation practices, folk taxonomy and economic potential was made. As many as 250 collections of wild mushrooms were made and worked out for their macro and micro-morphological features and ethnomycological details. Out of these, a total of forty four wild mushrooms distributed in 20 genera were found to be actually consumed in the state and possessed various potentialities. These includes the species of *Agaricus*, *Boletus*, *Clavaria*, *Clavulina*, *Coprinus*, *Geopora*, *Gyromitra*, *Helvella*, *Macrolepiota*, *Morchella*, *Otidea*, *Peziza*, *Pleurotus*, *Ramaria*, *Rhizopogon*, *Russula*, *Sepultaria*, *Sparassis*, *Termitomyces*, and *Verpa* (Table 1, Figure 1 & 2).

Table 1: Record of wild edible mushrooms commonly consumed and sold in the study area with range of palatability

S. No.	Species of fleshy fungi	Vernacular name	Consumed fresh/ dried	Market sale price in Rs/kg	Palatability range	
					P	M
1	*Agaricus arvensis*	Chaitar (2), Chaltee (1,3)	fresh	20-30	1	-
2	*Boletus edulis*	Bhutoo (1,3), Bhutol (1), Dailoo (1)	fresh	-	2	-
3	*Boletus granulatus*	Bhutoo (1,3), Dailoo (1)	fresh	-	2	-
4	*Boletus luridus*	Bhutol (1), Bhutoo (1,3), Dailoo (1)	fresh	-	1	-
5	*Clavaria vermicularis*	Shairee (1,3)	fresh	-	3	-
6	*Clavulina alpina* sp. nov.	Shairee (1,3)	fresh	-	3	-
7	*Clavulina cristata* var. *curta*	Shairee (1,3)	fresh	-	3	-
8	*Coprinus comatus*	Chaitar (2)	fresh	30-40	3	-
9	*Geopora arenicola*	Kundii (1,3), Kutch (2), Gav Padur (4), Khuduz (4), Kann Kutch (2,4)	fresh/dried	40-50	1	-
10	*Gyromitra esculenta*	Laal Thunthoo (1,3)	fresh	-	1	-
11	*Gyromitra gigas* var. *microspora* var. nov.	Cshitoo Thunthoo (1,3)	fresh	-	1	-
12	*Helvella atra*	Kaloo Thunthoo (1,3)	fresh	-	2	-
13	*Helvella crispa*	Thunthoo (1,3)	fresh	-	2	-
14	*Helvella elastica*	Thunthoo (1,3)	fresh	-	2	-
15	*Macrolepiota procera*	Chaltee (1,3)	fresh	20-30	3	-
16	*Morchella conica*	Thunthoo (1,3), Thunthoun (3), Pien Loj (2,4)	fresh/dried	5000-7000	1	+
17	*Morchella esculenta*	Thunthoo (1,3), Thunthoun (3), Batt Kutch (2,4)	fresh/dried	5000-7000	1	+
18	*Morchella semilibera*	Thunthoo (1,3), Thunthoun (3), Batt Kutch (2,4)	fresh/dried	5000-7000	1	+
19	*Morchella crassipes*	Thunthoo (1,3), Thunthoun (3),	fresh/dried	5000-7000	1	+

20	*Morchella deliciosa*	Thunthoo (1,3), Thunthoun (3),	fresh/dried	5000-7000	1	+
21	*Otidea leporina*	Nikdrii Kundii (1,3)	fresh	20-30	3	-
22	*Peziza badia*	Chaber Kann (1), Kann Kutch (2,4)	fresh	-	3	-
23	*Pleurotus squarrosulus*	Saroori (2), Shairee (1,3), Srij (2,4), Sirer (2)	fresh/dried	40-50	1	-
24	*Pleurotus* sp.	Saroori (2), Shairee (1,3), Srij (2,4), Sirer (2)			1	+
25	*Ramaria apiculata*	Shairee (1,3)	fresh	30-40	3	-
26	*Ramaria aurea*	Shairee (1,3)	fresh	30-40	3	-
27	*Ramaria flavobrunnescens* var. *aurea*	Shairee (1,3)	fresh	30-40	2	-
28	*Ramaria flavobrunnescens* var. *longisperma* var.nov.	Shairee (1,3)	fresh	30-40	2	-
29	*Ramaria formosa*	Shairee (1,3)	fresh	30-40	3	-
30	*Ramaria stricta*	Shairee (1,3)	fresh	30-40	2	-
31	*Rhizopogon guzmanii* var. *macrospora* var. nov.	Dudh Katt (1,3), Moraii Dudh Katt (1,3),	fresh/ uncooked	20-30	1	-
32	*Rhizopogon luteolus*	Haildu Dudh Katt (1,3), Moraii Dudh Katt (1,3), Matij (2,4)	fresh/ uncooked	20-30	1	-
33	*Rhizopogon luteolus* var. *multiguttulata* var. nov.	Haildu Dudh Katt (1,3), Moraii Dudh Katt (1,3), Matij (2,4)	fresh/ uncooked	20-30	1	-
34	*Rhizopogon sublateritus*	Haildu Dudh Katt (1,3), Moraii Dudh Katt (1,3), Matij (2,4)	fresh/ uncooked	20-30	1	-
35	*Russula* sp.	Babroo (1,3)	fresh	-	1	-
36	*Sepultaria sumneriana*	Kundii (1,3), Kutch (2), Gav Padur (4), Khuduz (4), Kann Kutch (2,4)	fresh/dried	40-50	1	-
37	*Sparassis crispa*	Bhedh Shairee (1), Rao Gaub (2,4), Rao Gabur (2,4)	fresh/dried	40-50	1	-
38	*Sparassis radicata*	Bhedh Shairee (1), Rao Gaub (2,4), Rao Gabur (2,4)	fresh/dried	40-50	1	-
39	*Sparassis cystidiosa*	Shairee (1,3)			3	-
40	*Termitomyces striatus*	Chaltii (1,3), Khumba, Chatree (5), Sootree (6)	fresh	40-50	1	-
41	*Termitomyces* sp.1	Chaltii (1,3), Khumba, Chatree (5), Sootree (6)	fresh	40-50	1	-
42	*Termitomyces* sp.2	Chaltii (1,3), Khumba, Chatree (5), Sootree (6)	fresh	40-50	1	-
43	*Termitomyces* sp.3	Chaltii (1,3), Khumba, Chatree (5), Sootree (6)	fresh	40-50	1	-
44	*Verpa conica*	Thunthoo (1,3), Thunthoun (3),	fresh/dried	5000-7000	1	-

Dialects:

1) Bhadarwahi, 2) Kishtwari, 3) Gaddaishi, 4) Kashmiri, 5) Dogri, = denotes not sold in the market

Palatability range:

1 = Delicious, 2 = Good, 3 = Just edible, M = Medicinal

Figure 1: **a** = Fruiting bodies of *Gyromitra esculenta,* **b** = Carpophores of *Gyromitra gigas* var. *microspora* var. nov., **c** = Fruiting bodies of *Helvella elastica,* **d** = Fruiting bodies of *Morchella conica* in natural habitat, **e** = Carpophore of *Morchella esculenta,* **f** = Carpophore of *Morchella semilibera,* **g** = *Morchella deliciosa* in natural habitat, **h** = Fruiting bodies of *Morchella crassipes,* **i** = Carpophore of *Verpa conica,* **j** = *Otidea leporina* in natural habitat, **k** = Fruiting bodies of *Geopora arenicola* in natural habitat, **l** = Development stages of *Sepultaria sumneriana,* **m-n** = *Rhizopogon luteolus* in natural habitat, **o, r** = Fruiting bodies of *Rhizopogon guzmanii var. macrospora* var. nov. in natural habitat, **p** = Fruiting bodies of *Rhizopogon luteolus* var. *multiguttulata* var. nov., **q** = Development stages of *Rhizopogon sublateritus.*

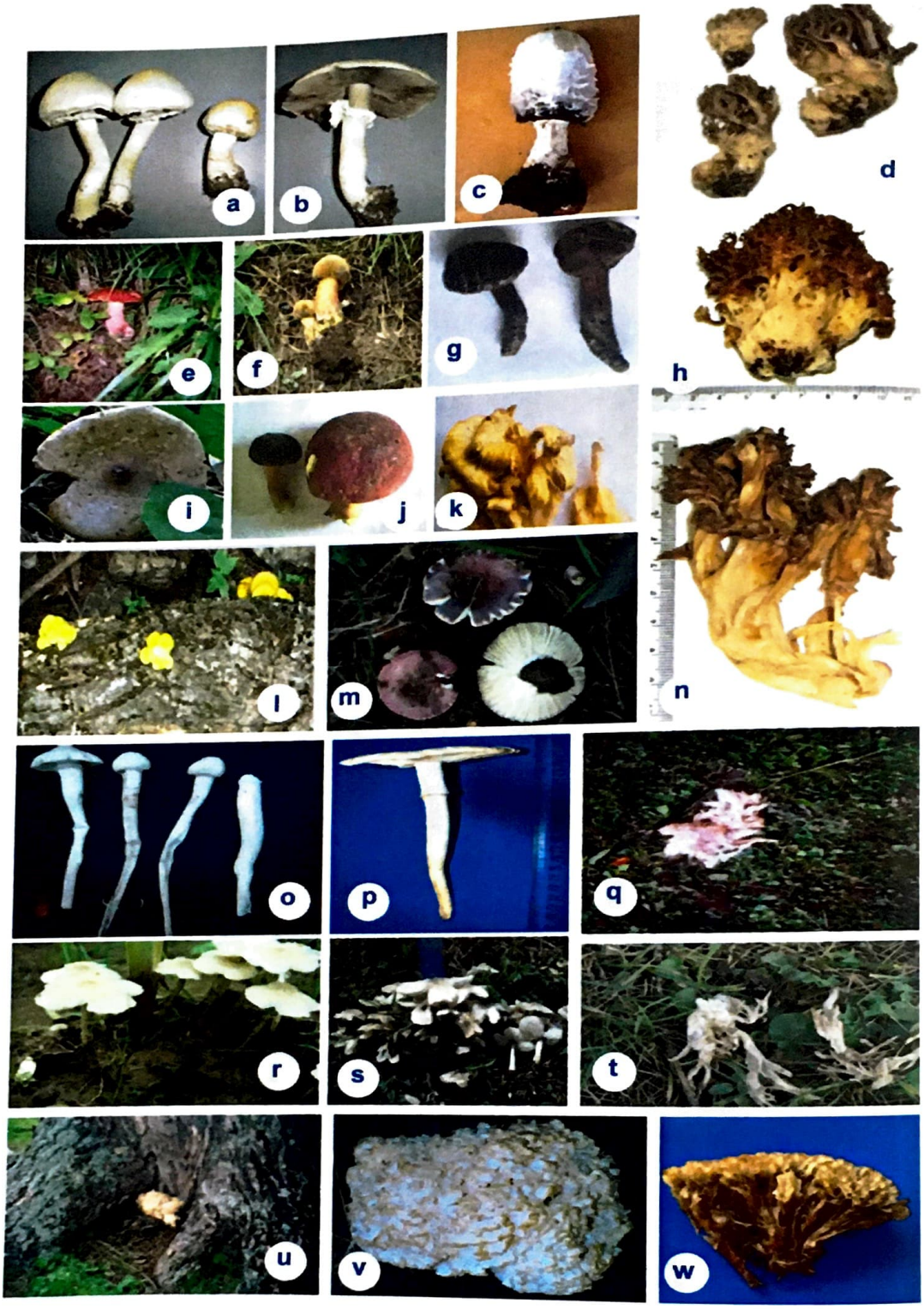

Figure 2: **a, b** = Fruiting bodies of *Agaricus arvensis*, **c** = Carpophore of *Coprinus comatus*, **d** = Fruiting bodies of *Ramaria formosa*, **e** = Carpophore of *Boletus luridus* in natural habitat, **f** = Carpophores of *Boletus edulis*, **g** = Carpophores of *Boletus granulatus*, **h** = Carpophore of *Ramaria flavobrunnescens* var. *longisperma* var. nov., **i** = Fruiting body of *Macrolepiota procera*, **j** = Carpophores of *Boletus* sp., **k** = Carpohores of *Pleurotus squarrosulus*, **l** = Fruiting bodies of *Pleurotus* sp. growing on *Juglans regia* in natural habitat, **m** = *Russula* sp. in natural habitat, **n** = Carpophore of *Ramaria aurea*, **o** = Development stages of *Termitomyces striatus*, **p** = Fruiting body of *Termitomyces* sp.1, **q** = Fruiting bodies of *Clavulina alpina* sp. nov., **r, s** = Fruiting bodies of *Termitomyces* spp. 2 & 3 in natural habitat, **t** = Fruiting bodies of *Clavulina cristata* var. *curta*, **u** = Carpophore of *Sparassis crispa* associated with *Cedrus deodara*, **v** = Fruiting body of *Sparassis radicata*, **w** = Carpophore of *Sparassis cystidiosa*.

Collection of wild edible mushrooms

Data gathered during the ethnomycological survey related to collection of wild mushrooms revealed that the collection of wild mushrooms was undertaken early in the morning, as there was intense competition for mushroom gathering, especially for the morels because of their high commercial value. Women and children from 'Gaddi', 'Shippi', 'Gujjar' and 'Pahari' tribes were frequently involved in these activities then men. Children frequently accompany the women, as they were good at locating mushrooms because of their sharp eyes and proximity to the ground and crevices where the occurrence of the mushroom was highest. A special basket called 'Tokri', 'Keed' or 'Chounllee' or a 'Cotton cloth' (Duppatta) was used for collecting mushrooms.

Collection forays were more frequent in March and April and July and August months. However, the best period for wild mushroom collection in the study area started with the onset of rains, the period when the conditions are conducive for the mushroom growth and they were available in plenty. This activity also coincided with the gathering of fallen pine needles used in roof topping of mud houses and firewood to be stored for winter months as the weather conditions during this period are harsh due to snow and fuel shortage.

Edibility Status

The ethnomycological survey related to edibility status of mushrooms was also undertaken and the results have been presented in Table 1. While confirming the edibility status of these mushrooms, the consumer's preference (range of palatability) was obvious (Table 1, Figure 3). Species such as *Agaricus arvensis*, *Boletus luridus*, *Geopora arenicola*, *Gyromitra* spp., *Morchella* spp., *Pleurotus* spp., *Rhizopogon* spp., *Sparassis crispa*, *S. radicata*, *Russula* sp., *Sepultaria sumneriana* and *Verpa conica* were considered highly delicious, while *Boletus edulis*, *B. granulatus*, *Helvella* spp., *Ramaria flavobrunnescens* var. *longisperma* var. nov., *R. aurea*, *R. stricta* had good acceptability for consumption. Remaining species namely, *Clavaria vermicularis*, *Clavulina* spp., *Coprinus comatus*, *Macrolepiota procera*, *Ramaria apiculata*, *R. flavobrunnescens* var. *aurea*, *Sparassis cystidiosa* and *R. formosa* were not much sought after mushroom species in the region.

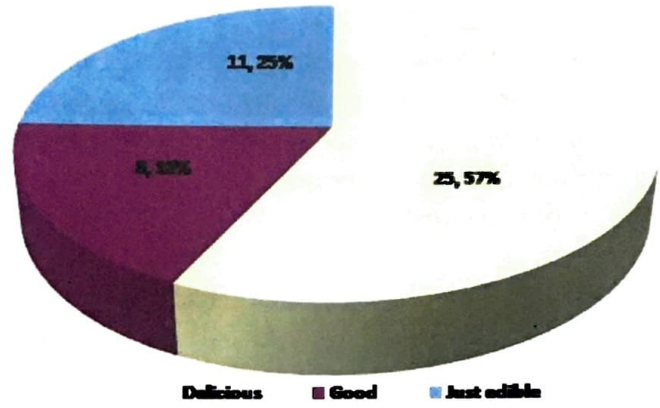

Figure 3: Representation of edible mushrooms with range of palatability in study area

Drying and Preservation

Fresh wild edible mushrooms have a short period during which they can be eaten or consumed. Owing to their perishable nature, they quickly deteriorate, rot, or shrivel up. On questioning local people about this aspect, it was realized that they consumed large number of the mushrooms in fresh form and only a few are preserved after sun-drying, smoke drying or salting. Mushroom species such as *Morchella* spp., *Pleurotus* spp., and *Sparassis* spp. were sun-dried in open and then stored in gunny bags, polythene bags or jars. In addition, a unique method for the preservation of cup fungi especially *Geopora arenicola* and *Sepultaria sumneriana* was followed by the locals. These hypogeous mushrooms were thoroughly washed in water to remove soil debris adhering to the apothecia, sun-dried, salted and then mixed with turmeric powder for enhancing shelf-life in storage and off-season consumption.

Culinary Potential

Outcome of the survey on the culinary potential of the edible mushrooms revealed that mushrooms in the study area represented a longed for culinary innovation and find a remarkable utility in the

culinary traditions of the area. Majority of the people questioned showed deep affection for the taste and preparation of wild mushrooms while only a few respondents showed aversion towards them. It was observed that the larger quantities of mushrooms were being consumed in the area and were regarded by many as wholesome food and in certain cases, an equivalent of meat. The inventory of consumption pattern of wild edible mushrooms extracted from the inhabitants of various regions of Jammu province of Jammu and Kashmir state especially Bhadarwah, Kishtwar, Poonch, Jammu, Ramban, Kathua, Rajouri, Bani, Udhampur etc was prepared. The mushrooms that were consumed as fresh vegetables included *Agaricus arvensis, Boletus* spp., *Coprinus comatus, Peziza badia, Clavaria vermicularis, Clavulina* spp. *Geopora arenicola, Gyromitra* spp., *Helvella* spp., *Macrolepiota procera, Morchella* spp., *Otidea leporina, Pleurotus* spp., *Ramaria* spp., *Sparassis* spp., *Sepultaria sumneriana, Russula* sp. and *Termitomyces* spp. These were usually cooked with tomatoes and onions while others were dried (*Morchella* spp., *Geopora arenicola, Sepultaria sumneriana, Sparassis* spp. *Pleurotus* spp., *Verpa conica* etc.) and consumed in off-season particularly during winter months during which the availability of vegetables is scarce in the hilly inhabitations and movement of the local people is restricted due to snowfall and harsh weather.

Several mushrooms (*Rhizopogon* spp) were eaten uncooked after cleaning and washing or while others were consumed after brief roasting on fire (*Boletus luridus* and *Russula* sp.). Likewise, the morels were used in making several traditional recipes prominent amongst which are 'Chaschni' (a local dessert), 'Thunthoo Pullow' (rice + morels), 'Thunthoo Kheer' (milk + morels), and 'Thunthoo Yakhni' (curd + morels). Therefore, the study indicates that wild mushrooms play an important dietary role as they are considered as a substitute of 'meat', a key ingredient in vegetables, flavouring agent or even as a condiment. Like spices and sauces, they could transform a routine monotonous diet into a feast.

Folk Taxonomy of wild edible mushrooms

Folk biological systems have been in practice throughout the world and they play an important role in local taxonomy. Research was carried out in various locations using five local dialects (Bhadarwahi, Kishtwari, Gaddaishi, Dogri and Kashmiri). Young informants, usually under the age of 25 years were able to recognize on an average 33% of the mushroom species while middle aged people (usually below 50 years and above 25 years) were familiar with nearly 50-60% of mushrooms, while the people in older age categories could distinguish maximum of collected wild mushrooms locally. Overall, women recognized more species as compared to men. This slightly greater recognition of mushrooms may be attributed to their greater involvement in collecting, cleaning and cooking of these mushrooms.

In English vernacular ethnomycology, mushrooms have been grouped as agarics, morels, puffballs, earthstars, truffles, coral fungi and many more based on their general morphology. Consequently, an extensive descriptive vocabulary relating to mushroom morphology, growth and habit was recovered during the interviews. It was inferred that the descriptive vocabulary used in the area was found to be remarkably comparable to the macro-morphological features used in scientific groupings of mushrooms.

As many as 35 vernaculars indigenously used for 44 wild mushrooms were recorded and transcribed. Some of the species were monotypic having only single names while others were polytypic i.e. having more than one vernacular. These vernacular names could be categorized based on gross morphology and life forms into main seven types or 'folk genera'. Most of these categories, inadvertently used though, referred to clearly defined biological groups such as 'Chaltee' or 'Sirer' for agarics; 'Bhutol', 'Bhutoo' or 'Dailoo' for boletes; 'Shairee', 'Gaub' or 'Gabur' for coral fungi. Similarly, puffballs were locally referred to as 'Dudh Katt'; earthstars as 'Sapp Nasvar'; morels as 'Thunthoo' and cup fungi were locally recognized by the folk genus 'Kundii'.

Many such significant contributions in the ethnomycological classification of the macrofungi

world over have been reported recently (Akpaja, et al., 2003; Ellen, 2008; Guissou, et al., 2008; Shepard Jr., 2008).

Common Beliefs and Practices Surrounding WEM Usage

Ethnomycological enquiries also threw light on broader social taboos, beliefs and famous remarks. Elderly men from 'Brahmin' or 'Pandit' community had strong aversion to mushroom consumption because of a 'strong taboo' fostered by folklores, which forbids Brahmins from eating mushrooms. Surprisingly though, it was observed that women and young children did not have any dissent towards mushrooms.

Species of *Sparassis*, *Ramaria* and *Clavulina* were consumed by the tribal (Gaddi, Shippi and Pahari) and poor families especially at dinner. Interestingly, the reason cited for this was that these wild mushrooms usually harboured worms (insect larvae) in the crevices of their branches and during the dark night hours children would not observe these worms (due to improper or no lightning in the remote areas). The elders assumed that these worms would be killed after cooking these mushrooms and thus, considered them safe for consumption. However, if these children, as they believed, spot these worms inside the fruiting bodies during daylight, they might develop strong aversion to these species which otherwise would be the cheapest source of their nutrition owing to their poverty.

Similarly, in several tribal hamlets viz. Chishoti, Hemoti, Kandail, Kansar, Sharaikhi, Patnitop, Bani, Sindra etc. it was commonly believed that those who collect the mushrooms, especially morels, should not eat them because once they do so, they might lose the power to search and locate them and thus would not find magical mushrooms anymore. Though no rationale is known behind this belief, it might be probably due to the fact that since the trade value of morels is very high, the mushroom collectors could reduce their intake so as to earn cash to sustain their other family needs.

One of the myths related to morels was that a single small morel around the neck could ward off the bad souls.

Traditional Remarks Based on Some Wild Mushrooms

Few common remarks for the presence of some edible species such as morels in the forest locations of the study area also came to our knowledge. For instance:

'Dhouns Bazzay Beinsh Bazzay, Bin Beezzay Rukh Zamaaye'

(Meaning: Drums and flutes are not there, still the music is aloud, Thick cloud and heavy rains sprout the mushrooms (morels) out of the earth).

The love and affection of people for some of the delicious mushrooms is clearly depicted by the personification of the cup fungi (*Geopora arenicola and Sepultaria sumneriana*). And the sayings go like:

'Paar Banay Maire Bhabi Bassay, Main Jaoun Tey Khir Khir Hassay'

(Meaning: Across there in forest, my sister-in-law resides, Who, on my visit, greets me with many smiles), and

'Paar Banay Maira Bhaiya Bassay, Main Jaoun Tey Khir Khir Hassay'

(Meaning: Across there in forest, my brother resides, Who, on my visit, greets me with many smiles).

In conclusion, the study indicates the cultural importance and long traditional use of wild mushrooms in Jammu and Kashmir. Further investigations for thorough, careful and comprehensive forays for ethnomycological facets of this important group of organisms existing in various locations of the state are called for. The study also recommends regular surveys over an extended period in order to assess the patterns of abundance of mushrooms in different seasons. From such information, harvesting strategies and management plans can be formulated and implemented to ensure the lasting presence of these socially and economically important species. This becomes even more relevant when Food and Agricultural Organization has recommended the

use of edible mushrooms as food supplement for protein deficient populations of developing and underdeveloped countries.

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Characterization of Rice [*Oryza sativa* L.] Germplasm Based on Iron and Zinc Content

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Abstract
Hidden hunger is caused by the consumption of food grains (rice) which are deficient in mineral micronutrients specifically iron and zinc. This hunger affects more than one-half of the world's population especially woman and children in developing countries causing anemia and child blindness because 60 per cent of the world's population is dependent on rice as their staple food. Considering this biofortification as one of the suitable approaches was considered for improving the Fe and Zn content and their bioavailability in rice grain. Local landraces of rice were collected and Fe and Zn content were quantified for their genotypic characterization. Iron (Fe) and Zinc (Zn) content of 112 local landraces were estimated according to Lindsey and Norwell by using Atomic Absorption Spectrophotometer Varian Speetra AA 50B. Iron concentration ranged from 0.25µg/g to 3.25µg/g and zinc from 0.85µg/g to195.3µg/g in the landraces. Highest iron containing rice was Swetonunia with 34.8µg/g and highest Zn was found in Nepali Kalam which was 195.3µg/g. Zn content is quite comparable to other HYVs and can be used as donor parent in breeding program for biofortification of micronutrient Zn.

Keywords: Rice landraces, Minerals Iron, Zinc, and Genotyping.

Introduction

Rice is a major staple food and energy source of more than half of the world population, being the major source of carbohydrate and even protein. However, rice is a poor source of essential micronutrients such as Fe and Zn (Bouis and Welch 2010). In countries where rice is used as staple food, the per capita consumption is very high ranging from 62 to 190 kg/year. Thus, even a small increase in the nutritive value of rice can be highly significant for human nutrition (Grahama *et al.* 1999). Micronutrient malnutrition, and particularly Fe and Zn deficiency affect over three billion people worldwide, mostly in developing countries (Sperotto *et al.* 2010). Diet deficient in minerals such as Fe and Zn in staple food crops causes 'hidden hunger' or micronutrient malnutrition in developing countries (Welch *et al* 2004). It causes several diseases (anemia, endemic goiter, child blindness, *etc.*); the affected people are more prone to infection to other diseases resulting in further deterioration in quality of life. Of these, iron deficiency is the most common nutritional disorder in the world affecting over 4 billion people, with more than 2 billion woman, mainly in developing countries (WHO; http://www.who.int/nut/ida.htm). Zinc deficiency in humans reduces growth, sexual maturity and the immune defense system (Parsad 1993). The human body requires more than 22 mineral elements that can be supplied by an appropriate diet (Philip and Martin 2005). Trace minerals are important not only for human nutrition, but for plant nutrition as well, plant breeding holds great promise for making a significant, low-cost, and sustainable contribution to reducing micronutrient, particularly mineral deficiencies in humans, and may have important by-product effects for increasing farm productivity in developing countries in a way that is environmentally-beneficial (Cary *et al.* 1994, Kannenberg *et al.* 1995).

Several groups have examined the probability of "Biofortification" approach for improving the micronutrient (iron and zinc) content of staple crops including rice. It is observed that substantial useful genetic variation exists in key staple crops. Nutritional quality traits are highly heritable in some crops, mineral rich traits are sufficiently stable across a wide range of growing environments, and

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traits for high micronutrient content can be combined with superior agronomic and high yield characteristics.

Most of the commercially cultivated indica and japonica rice cultivars are deficient in iron and zinc compared to the other staple food crops such as wheat and maize (Gregorio *et al.* 2000). Zinc deficiency is probably the most widespread micronutrient deficiency in cereals. Sillanpaa (1990) found that 49 percent of the global sample of 190 soils in 25 countries was low in zinc. Unlike other micronutrients, zinc deficiency is a common feature of both cold and warm climates, drained and flooded soils, acid and alkaline soils, and both heavy and light soils (Rahman *et. al.* 1993).

Since rice is the principal food of the Asian continent (Developing world), a lot of efforts are being made to develop nutritionally improved genotypes of rice (Sasaki 1998). The first pre-requisite for initiating a breeding program to develop micronutrient-rich genotypes, is to screen the available germplasm and identify the source of genetic variation for the target trait, which can be used in crosses, genetic studies, molecular marker development and to understand the basis of enhanced micronutrient accumulation. Available literature do show existence of variability for grain iron and zinc contents in rice, but to date only a small portion of the existing genetic diversity has been assayed for micronutrients (Gregorio *et al.*1999).

A distinction has to be made between content and concentration. The content of iron and zinc in rice depends on the grain size. Aromatic long grain basmati lines are known to be high in iron content. The high or low content of mineral elements in grain largely determine the nutrient value of rice. Zhang *et al* (2005) showed that single grain selection of narrow grains tends to increase the content of Zn, Mn and P, while selection of single plants with bigger grain weight tends to increase the content of P.

Considering all the above references, objectives of the present investigation is to screen rice germplasm for iron and zinc content.

Materials and Methods

Rice cultivars

Total of 112 rice cultivars were collected from West Bengal and adjoining area (during kharif 2010 & 2011) and maintained at the Plant Genetics & Tissue Culture Laboratory, University of North Bengal (NBU), India.

Fe and Zn concentration analysis

Iron (Fe) and Zinc (Zn) content of 112 local cultivars were estimated according to standard method (Lindsey and Norwell 1969) by Atomic Absorption Spectrophotometer (Varian Spectra AA50B). Seeds from all varieties were dehusked gently using a palm dehusker. Concentration was expressed in µg/g. One gram oven dried ground dehusked seed samples were collected in a 150 ml conical flask. To this 25-30 ml diacidic mixture (HNO3:HClO4; 5:1 v/v) was added and kept overnight. Next day, it was digested by heating till clear white precipitates settled down at the bottom. The crystals were dissolved by diluting in double distilled water. The contents were filtered through Whatman No. 42 filter paper. The filtrate was made to 50 ml with double distilled water. The acid digested samples were used for the determination of iron and zinc contents.

Results and Discussion

Iron content ranged between 0.25µg/g to 3.25µg/g and Zinc ranged between 0.85µg/g to 195.3µg/g (Table 1). Local cultivar Swetonunia had highest iron content of 34.8µg/g followed by the other cultivars Chamormoni 3.25µg/g, Bunkulon 3.15µg/g, Govindobhog 3.1µg/g, and Addey 2.05µg/g (Table 1). Nepali Kalam had the highest Zinc content 195.3µg/g followed by Govindobhog 138.6 µg/g, Begunbeej 20.4µg/g and Ghiosh16.15µg/g. Iron content in all the local landraces were very poor but Zinc content in some of the landraces was promising containing 195.3µg/g in Nepali Kalam and 138.6µg/g in Gobindobhog).

On the basis of iron contents, rice cultivars could be grouped in two categories, low (0-10µg/g); moderate (>10µg/g). Similarly, rice cultivars were placed into two groups on the basis of zinc contents

low (<10μg/g); moderate (>10μg/g). Most of the rice cultivars studied here was placed in the low iron and zinc containing categories. Least amount iron content cultivars are Birohi (0.4μg/g), Kabiraj (0.35μg/g), and Tulaipanji (0.45μg/g). Similarly least amount zinc content cultivars are Birohi (0.85), Chamormoni (0.85μg/g) and Thulo Addey (1.4μg/g). These results indicated that there is significant genetic diversity in the rice germplasm.

A plant breeding strategy has been formulated to improve the mineral nutrition in rice grain which includes selecting for germplasm with greater quantities of essential minerals (such as Fe, Zn etc). For this the breeding lines that are with high yields and accumulate minerals from infertile soils are selected and enhancing bio-available minerals in edible portions through increasing the concentrations of metal-binding proteins (Fumiyuki *et al.* 1999; Lucca *et al.* 2001; Holm *et al.* 2002; Zhang *et al.* 2004; Heinemann *etal.* 2005; Philip and Martin 2005).

Of 112 rice genotypes evaluated, one genotype, Swetonunia was placed under high iron category 34.8μg/g and Nepali Kalam under high Zinc 195.3μg/g category. Grahem *et al* (1999) and Gregorio *et al* (2000) reported wide range of Fe (6.3-24.4μg/g) and Zn (13.5-58.4μg/g) concentration in brown rice. Notably, highest grain-Fe (18-22μg/g) and grain-Zn (24-35μg/g) concentration were found in several aromatic rice varieties such as Jalmagna, Zuchen and Xua Bue Nuo. Notably, there was about many fold difference in Fe and Zn content suggesting the existence of genetic potential to increase the concentration of these micronutrients in rice grain. This type of large genotypic variation especially for iron content in rice has not been reported earlier. Iron and Zinc contents in edible portions also depend on the efficiency of translocation of minerals from root tissues to edible plant organs and accumulation thereof. Mineral-rich and mineral poor rice genotypes identified in this study may be used in breeding program for introgression of high Fe and Zn content gene or QTLs in the improved varieties.

Table 1. Iron and Zinc content in the rice germplasm

Sl. No.	Rice landraces	Fe (μg/g)	Zn (μg/g)
1	Agundhepi	2.05±0.011	9.77±0.011
2	Aichung	4.3±0.003	3.8±0.029
3	Anandi	0.75±0.025	2.55±0.003
4	Ashami	0.7±0.029	3.4±0.003
5	Attey-1	7.9±0.006	4.1±0.002
6	Attey-2	6.9±0.002	1.0±0.011
7	Badsabhog	6.2±0.001	2.1±0.008
8	Banni	6.9±0.002	1.4±0.001
9	Begunbeej	7.0±0.002	2.1±0.008
10	Bhadaore	1.6±0.021	20.4±0.020
11	Bunkulon	7.3±0.001	2.8±0.005
12	Chamormoni	3.15±0.008	10.62±0.58
13	Champa	3.25±0.068	0.85±0.28
14	Champasali	0.6±0.024	2.55±0.21
15	Champasari	5.2±0.004	2.4±0.003
16	Chanachur	0.96±0.015	8.8±0.104
17	Charinagrey	6.8±0.004	1.5±0.001
18	China Boro	4.7±0.005	2.6±0.001
19	Chiniatop	0.33±0.032	3.4±0.24
20	Chinisakkar	13.4±0.008	4.4±0.003
21	Chirakhe	6.0±0.000	2.3±0.001
22	Chulthey	4.5±0.007	2.12±0.097
23	Chunakathi	7.0±0.000	3.4±0.004
24	Chunia	7.1±0.003	2.1±0.003
25	Dangimarua	7.3±0.001	2.7±0.002
26	Desi nunia	6.7±0.002	2.5±0.009
27	Dhankutte	7.0±0.004	3.2±0.004
28	Dhanraj	4.7±0.011	2.7±0.000
29	Dhepi	4.9±0.002	2.2±0.008
30	Dhusuri dhan	7.0±0.002	2.3±0.13
31	Kalamkathi	4.6±0.002	1.7±0.13
32	Kalampanati	4.9±0.001	2.4±0.002
33	Kalobhog	7.1±0.001	2.4±0.12
34	Kalojera	6.9±0.001	1.6±0.003
35	Kalokure	10.7±0.002	1.9±0.16
36	Kalonunia	6.0±0.005	2.55±0.266
37	Kakuriya	7.0±0.004	4.1±0.14
38	Kanta Rangi	4.9±0.002	2.6±0.009
39	Kantajinghasal	3.8±0.003	3.9±0.003
40	Kataribhog	5.5±0.001	2.6±0.009
41	Kattaka	5.1±0.003	2.5±0.006
42	Khalkhajara	6.9±0.001	2.5±0.009
43	Khasa dhan	6.9±0.004	2.2±0.001
44	Khasdhan	4.0±0.002	2.1±0.11
45	Kholako Dhan	0.25±0.07	2.12±0.385
46	Khechri	1.73±0.014	4.67±0.137
47	Koshia Binni	4.3±0.001	2.1±0.017
48	Kumrogore	7.2±0.001	1.6±0.007
49	Ladua	8.0±0.001	2.9±0.006

Sl. No.	Rice landraces	Fe (µg/g)	Zn (µg/g)
50	Lalmala	5.4±0.008	2.4±0.004
51	Ravan	12.1±0.001	6.7±0.001
52	Sadanunia	7.8±0.002	35.2±0.002
53	sanu addey	6.9±0.002	2.2±0.004
54	Sikkimey	6.7±0.005	2.7±0.18
55	Sitabhog	9.7±0.002	6.3±0.14
56	Sorulalat	11.9±0.001	3.2±0.006
57	Bhadoi	0.5±0.038	1.7±0.002
58	Bhale musuri	3.0±0.004	1.7±0.002
59	Bhangeri	6.7±0.000	1.7±0.001
60	Bharlang	6.7±0.001	1.8±0.003
61	Borni	6.9±0.004	2.3±0.008
62	Borisal	4.2±0.011	2.5±0.016
63	Birohi	0.70±0.05	7.6±0.105
64	Birimphole	0.4±0.026	0.85±0.70
65	Banni	7.1±0.003	3.3±0.004
66	Buchi	7.0±0.002	2.1±0.008
67	Dos nunia	6.9±0.004	3.8±0.001
68	Dudhekalam	4.4±0.001	2.0±0.008
69	Dudheswar	5.8±0.005	2.4±0.005
70	Dudhey	65.4±0.055	8.5±0.008
71	Enda	0.4±0.021	0.85±0.35
72	Ghiosh	0.55±0.13	8.5±0.177
73	Gokhraj	8.6±0.002	2.0±0.006
74	Govindobhog	6.2±0.002	138.6±0.071
75	Harintore	12.8±0.005	1.4±0.001
76	Hipsa nunia	6.9±0.001	2.9±0.009
77	IR64	5.00±0.003	2.0±0.12
78	Jaldhepa	0.5±0.029	1.27±0.199
79	Jamaisal	7.0±0.002	3.2±0.006
80	Jeerasare	4.4±0.001	2.0±0.006
81	Jetti dhan	4.4±0.004	1.9±0.11
82	Jhapka	5.2±0.003	2.1±0.002
83	Jhulur	6.5±0.002	4.6±0.14
84	Jungli	1.8±0.023	15.4±0.077
85	Kaberi	7.1±0.002	1.8±0.11
86	Kabiraj	0.35±.049	0.85±0.99
87	lalpanati	8.3±0.009	3.2±0.008
88	Laxmansal	6.8±0.004	1.3±0.003
89	Laxmikajal	7.3±0.001	2.5±0.008
90	Magursali	7.0±0.003	2.9±0.007
91	Mala	6.6±0.003	4.2±0.007
92	Malsiara	7.0±0.003	1.7±0.004
93	Minjurijal	7.1±0.003	2.9±0.003
94	Murshi	9.8±0.003	1.5±0.006
95	Nageswari	6.6±0.004	2.2±0.11
96	Nagra	6.3±0.003	2.4±0.003
97	Nav dhan	6.6±00.03	1.7±0.006
98	Nazarius Ekka	6.8±0.000	2.5±0.14
99	Nepali Kalam	12.3±0.001	195.3±0.3
100	Pahal man	6.7±0.001	2.0±0.005
101	Paijam	5.2±0.001	1.7±0.007
102	Pari	4.8±0.006	2.8±0.009
103	Phoolpakri	7.4±0.003	3.4±0.001
104	Puasabasmoti	6.2±0.004	1.9.009
105	Radhunipagal	6.2±0.003	2.0±0.005
106	Rasna	6.9±0.003	1.7±0.007
107	Swetonunia	34.8±0.003	35.2±0.002
108	Tangrey	0.43±0.06	1.27±0.34
109	Thakurbinni	6.4±0.008	3.0±0.001
110	Thulo addey	5.1±0.001	1.4±0.006
111	Timbure	13.8±0.001	4.2±0.001
112	Tulaipanji	0.45±0.04	2.21±0.14

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Isolation and identification of a virulent *Ralstonia solanacearum* by *fliC* gene amplification and induction of chitinase by 2-amino butyric acid for control of bacterial wilt in tomato plants

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Abstract
Ralstonia solanacearum is a devastating, soil borne bacterial pathogen of tomato. The pathogen is nonmotile in planta but highly motile in culture. On the basis of physiological and biochemical characteristics 26 isolates have been purified and identified as *Ralstonia solanacearum*. The *fliC* gene is responsible for the movement of bacteria. *Ralstonia* specific *fliC* gene amplification is the indication of virulence of the pathogen. In the present study one *R. solanacearum* isolate has been identified by PCR amplification of the *fliC* gene using *fliC* gene specific primer. Following isolation and identification of the virulent isolate, fresh tomato plants were induced by application of 2-amino butyric acid (ABA). The defense enzyme, chitinase was estimated in treated plants. Treated inoculated plants did not show any visible symptoms of wilt even after 14 days of inoculation. Significantly it was observed that chitinase was increased in the 2-ABA-treated plants and also in the treated-inoculated plants. The increased chitinase activity in the treated plants showed that 2-ABA has the resistance inducing capacity in tomato plants against *Ralstonia solanacearum*.

Keywords: Isolation, *Ralstonia solanacearum*, *fliC* gene, chitinase, 2-ABA.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most economically important vegetable crop and it is cultivated worldwide for its freshly fruits and economic importance (Elphinstone *et al.*, 1996). The causal organism of bacterial wilt is *Ralstonia solanacearum*. *R. solanacearum* is a soil born bacterium originating from the tropics, subtropics and warm temperate regions (Hayward, 1991). *R. solonacearum* is a serious pathogen causing bacterial wilt in solanaceous vegetables in India, such as tomato, potato, banana, eggplants and some ornamental plants (Tans-Kersten *et al.*, 2001).

Control of bacterial wilt is difficult and it may be done by using resistant cultivars. Chemical control is not suitable and use of fumigants is of limited use. Biological control has also been suggested by some authors (Goellner and Conrath, 2008).

In the genomic DNA of the bacterium the *fliC* gene is found. *fliC* gene is responsible for the production of flagellin protein, which has a major role in the movement of bacteria. Presence of *fliC* gene also indicates the virulent nature of the bacteria (Pfund *et al.*, 2004). Hence, if amplification of the *fliC* gene could be done by using *fliC* gene primers, the virulent nature of the isolate may be ascertained. The present work has been undertaken to isolate virulent *Ralstonia solanacearum* isolates from infected plants of north Bengal by PCR amplification of *fliC* gene visible on the agarose gel. After identification of a virulent bacterium, its management becomes necessary. The most virulent isolate of the present study, therefore, controlled by inducing defense enzyme, chitinase, in susceptible tomato plants by 2-ABA.

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MATERIALS AND METHODS

Survey of different diseased tomato fields of North Bengal

The infected plant samples were collected from different tomato growing fields of North Bengal. The three districts (Uttar Dinajpur, Dakshin Dinajpur and Cooch Behar) were chosen for the present study.

Isolation of bacterial isolates from diseased plants

The infected plant samples were collected and brought to the laboratory for the isolation of the bacteria. Then all plant samples were washed with distilled water and a cross section of each diseased plant stem was made, which produced white, milky layer of bacterial cells (bacterial ooze) in clear sterile distilled water (Fig. 1). Then the water was used as sample for isolation of bacteria (Leppla *et al.*, 2004) 0.1 ml of bacterial suspension was spread

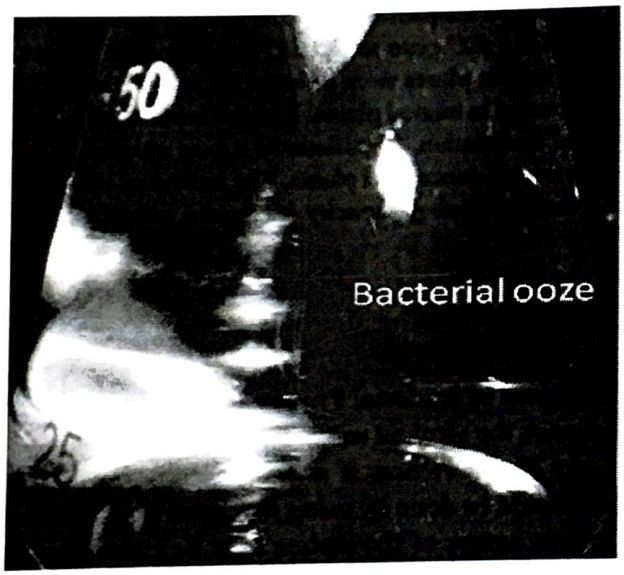

Figure 1. Bacterial ooze coming out from the cut end of stem of infected tomato plants.

on *Pseudomonas solanacearum* agar medium and incubated at 32° C for 24 hours. After the incubation the bacterial isolates were subcultured in fresh *Pseudomonas solanacearum* agar medium. All together 26 such samples were found to be pure and kept in the laboratory with proper code for further use in future.

Biochemical characterization

According to the Lacy and Lukezic, (2004) several biochemical tests were performed. The biochemical tests were Gram staining, anaerobic growth, yellow pigmentation on YDC medium, growth above 40°C, growth below 4°C, growth on D1M medium and oxidase test.

DNA extraction and PCR amplification

The method of Ausubel *et al.*, (1992) was followed for the isolation of genomic DNA. Polymerase chain reaction (PCR) was performed using a set of *fliC* gene primer as suggested by Schonfeld *et al.*, (2003).

Primer pair: Rsol_*fliC* forward 5′-GAACGCCAACGGTGCGAACT-3′

and reverse 5′-GGCGGCCTTCAGGGAGGTC-3′.

Assay of chitinase

The colorimetric assay of chitinase was carried out according to the procedure developed by Mahadevan and Sridhar, (1982). Colloidal chitin was prepared as per the method of Berger and Reynolds, (1958). One gram of tomato leaves were extracted in 5.0 ml of 0.1M sodium acetate buffer, pH 5.2 containing 700mg of PVP using mortar and pestle in cold condition. The homogenate was filtered by using four layered muslin cloth, centrifuged at 10000g for 10 minutes and the supernatant was used as crude enzyme source. The assay mixture consisted of 0.5ml crude enzyme, 0.25ml of 0.1M sodium acetate buffer, pH 5.2 and 1ml colloidal chitin (1.8mg/ml) incubated at 37°C for 2h. One ml of reaction mixture was taken and then 1ml of distilled water was added to it. The mixture was boiled for 10 minutes and centrifuged at 5000g for 3 minutes to stop the reaction. One ml of the supernatant was added to 0.1ml of 0.8M potassium tetra borate and boiled exactly for 3 minutes. Then hot mixture was cooled and added to 3ml of para-di-methyl amino benzaldehyde (DMAB) reagent. Samples were incubated again at 37°C for 20 minutes. Immediately after incubation the mixture was cooled and absorbance was recorded within 10 minutes at 585 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis ($\mu mol\ min^{-1}\ g^{-1}$) using N-Acetyl-D Glucosamine as standard.

RESULTS AND DISCUSSION

On the basis of visual observation, 26 bacterial-wilt affected samples were collected from three districts of North Bengal. All the samples were

brought to the laboratory and bacteria were isolated from each sample following the technique as described in materials and methods. All the bacteria were coded. After the isolation of the bacteria, they were allowed to infect tomato plants grown in the experimental plots. Pathogenicity of each bacterium was separately assessed and the wilting index was calculated on the basis of a five point scale. Results of the pathogenicity have been presented in table-1. From the results (Table-1) it was evident that *R. solanacearum* (isolate T_6) of Haldibari of Coochbehar district was most virulent and caused complete wilting (wilting index = +++++) of the test plants. Thirteen isolates showed least wilting index (+). Twelve isolates showed moderate wilting.

Table 1: Isolation of bacteria from infected plants of the places studied

Sr. No.	Place of collection of diseased samples	Isolate code	Pathogenicity status (Wilting Index)
1	Haldibari Cooch Behar)*	T_1	++
2	Haldibari (Cooch Behar)	T_4	++
3	Haldibari (Cooch Behar)	T_5	+
4	Haldibari (Cooch Behar)	T_6	+++++
5	Haldibari (Cooch Behar)	D_1	++
6	Haldibari (Cooch Behar)	D_2	+
7	Haldibari (Cooch Behar)	D_3	++
8	Haldibari (Cooch Behar)	D_4	++
9	Haldibari (Cooch Behar)	D_5	+
10	Haldibari (Cooch Behar)	D_6	+
11	Durgapur (Uttar Dinajpur)	A_1	+
12	Durgapur (Uttar Dinajpur)	A_2	++
13	Durgapur (Uttar Dinajpur)	A_3	+
14	Durgapur (Uttar Dinajpur)	A_4	+
15	Balurghat (Dakshin Dinajpur)	G_1	+
16	Balurghat (Dakshin Dinajpur)	H_1	+
17	Balurghat (Dakshin Dinajpur)	H_4	++
18	Balurghat (Dakshin Dinajpur)	H_5	++
19	Balurghat (Dakshin Dinajpur)	H_6	+
20	Balurghat (Dakshin Dinajpur)	H_7	+
21	Balurghat (Dakshin Dinajpur)	H_8	++
22	Balurghat (Dakshin Dinajpur)	H_9	++
23	Balurghat (Dakshin Dinajpur)	H_{10}	++
24	Ghoksadanga (Cooch Behar)	S_1	+
25	Ghoksadanga (Cooch Behar)	S_2	+
26	Ghoksadanga (Cooch Behar)	S_3	++

*Names given in parentheses are the name of the districts of the collection spot.

Biochemical characterization

All the isolates were subjected to biochemical characterization for identification. Results of the Gram reaction showed that all the 26 bacteria were Gram negative. From the other results (Table-2) it was also evident that all the bacteria were aerobic. Two bacteria (isolate S_3 and D_5) could produce pigment on YDC medium and the other 24 bacteria were unable to produce pigment. Only two bacteria (isolate D_4 and H_5) could grow at 40° C and above. But, no bacteria could grow at 4°C. One bacterium (isolate D_4) could grow on D1M medium. Out of the bacteria tested three bacterial isolates (isolate H_4, H_5 and A_3) were oxidase negative. The above results were compared with that of given in Bergey's Manual of systematic bacteriology, Vol 1, section 4. Compared results suggested that all the 26 bacteria were *Ralsonia solanacearum.* Our

Table 2: The biochemical characteristics of the isolated bacteria

Isolates in code	Biochemical tests performed						
	Gram staining	Anaerobic growth	Yellow pigmentation on YDC media	Growth above 40°c	Growth below 4°c	Growth on D1M medium	Oxidase test
S_1	-	-	-	-	-	-	+
S_2	-	-	-	-	-	-	+
S_3	-	-	+	-	-	-	+
D_1	-	-	-	-	-	-	+
D_2	-	-	-	-	-	-	+
D_3	-	-	-	-	-	-	+
D_4	-	-	-	+	-	+	+
D_5	-	-	+	-	-	-	+
D_6	-	-	-	-	-	-	+
G_1	-	-	-	-	-	-	+
H_1	-	-	-	-	-	-	+
H_4	-	-	-	-	-	-	-
H_5	-	-	-	+	-	-	-
H_6	-	-	-	-	-	-	+
H_7	-	-	-	-	-	-	+
H_8	-	-	-	-	-	-	+
H_9	-	-	-	-	-	-	+
H_{10}	-	-	-	+	-	-	+
T_1	-	-	-	-	-	-	+
T_4	-	-	-	-	-	-	+
T_5	-	-	-	-	-	-	+
T_6	-	-	-	-	-	-	+
A_1	-	-	-	-	-	-	+
A_2	+	-	+	-	-	-	+
A_3	-	-	+	+	-	-	-
A_4	+	-	+	-	-	-	+

results were also compared with the flow chart identification of of genera of phytopathogenic bacteria as suggested by Lacy and Lukezic,2004

Confirmation of virulence of the bacterial isolate T6 of *R. solanacearum*

Virulance of *R. solanacearum* has been correlated with the presence of *fliC* gene in the bacterium. In the present study the presence of the gene has been confirmed by amplification of the *fliC* gene by PCR and visualization of the amplicon of the expected size (. 550bp) on agarose gel after electrophoresis (Fig. 2). The expected amplicon confirmed the virulence of the bacterium shown during pathogenicity test. The presence of *fliC* gene in the present virulent isolate are in conformity with that of Pfund *et al.*, (2004) who reported that for full virulence flagellin, a product of *fliC* gene is required.

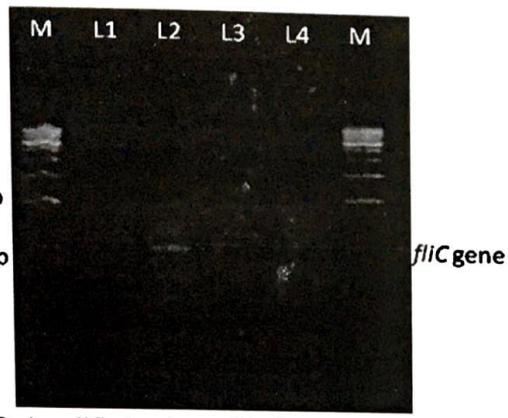

Figure 2. Amplified prduct of *fliC* gene of the virulent isolate T_6 on agarose gel after electrophoresis.

Induction of defense enzyme: chitinase

Many phytopathogenic fungi contain chitin as major structural cell wall component (Wessels and Sietsma, 1981). Chitinases commonly known as plant hydrolases are the key defense enzyme for plant protection against fungal pathogens. Several bacteria were also controlled by induction of chitinase in host plants. Although chitinase degrade chitin present in fungal wall components and several authors have demonstrated the activity of chitinase as growth inhibitor of fungi (Arlorio *et al.*, 1992; Mauch *et al.*, 1988), but the mechanism of controlling bacterial pathogen are yet to be known. In the present study the defense enzyme chitinase have been induced by 2-ABA treatment. From the results presented in Table 3 it was evident that inoculated plants showed maximum chitinase activity (22mg GlcNAc g^{-1} tissue h^{-1}) at 9th day but 2-ABA treated plants showed maximum activity (60mg GlcNAc g^{-1} tissue h^{-1}) after the same period of treatment. When simultaneous treatment and inoculation were done more chitinase activity (65mg GlcNAc g^{-1} tissue h^{-1}) were observed. In distilled water treated plants chitinase activity was recorded as 14mg GlcNAc g^{-1} tissue h^{-1}. From the results it may be concluded that induction of chitinase activity is related to control of the bacterial pathogen in tomato. Chitinase has been reported to control *R. solanacearum* and some other Gram negative bacteria by agglutination (Guan *et al.*, 2008). Thus increased level of chitinase in plants inhibits the entry of the virulent bacterial pathogens in plant cells and thus protects the plants.

Table 3: Chitinase activity in tomato (variety PKM 1) pretreated with 2-ABA followed by challenge inoculation of *Ralstonia solanacearum*

Plant treatment	Chitinase activity (mg GlcNAc g^{-1} tissue h^{-1})			
	Days after treatment and /or inoculation			
	3 Days	6 Days	9 Days	12 Days
Pathogen inoculated	12	15	22	20
Treated with 2-ABA	30	47	60	55
Plants pretreated with 2-ABA and inoculated with *R. solanacearum*	35	55	65	60
Plants sprayed with distilled water (Control)	0.5	0.8	14	10

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