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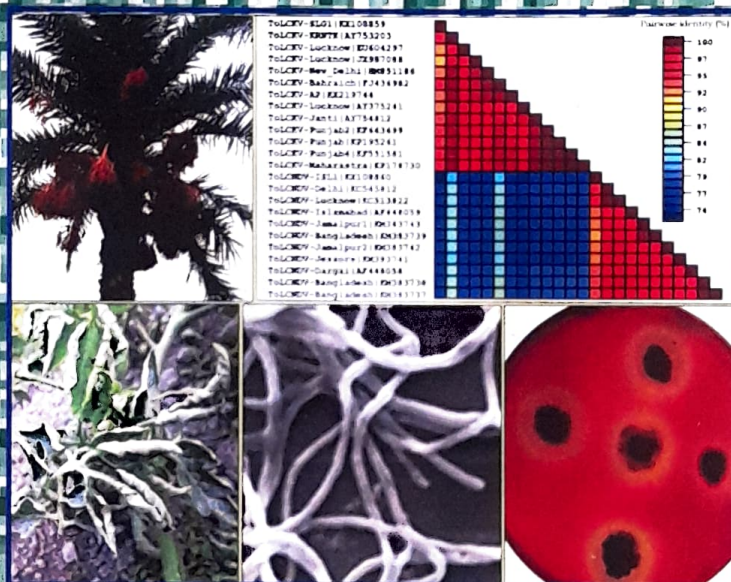

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Review Article
Copper toxicity in plants: a review and a case study on tea

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Abstract

Copper in trace amounts is essential for various metabolic processes in the plant such as photosynthesis, carbohydrate distribution, and protein metabolism but at high concentration it causes physiological stress through generation of free radicals that induce the production of reactive oxygen species (ROS) via Haber-Weiss and Fenton reactions. Copper-induced generation of hydrogen peroxide, hydroxyl radicals, or other reactive oxygen species has been directly correlated with the damage to protein and lipids that may lead to reduced growth and even death. Tea (*Camellia sinensis* (L.) O. Kuntze) is an economically important plantation crop in India with round the year productivity. Copper based fungicides are cheap and effective in controlling fungal diseases and are used consistently throughout the year to combat different fungal diseases that pose a major threat to tea production. Excess Cu^{2+} has been found to alter several physiochemical parameters in the tea plants. A more detailed study on mechanisms of Cu^{2+} toxicity at the gene level is warranted.

Key words: Copper, stress, tea, reactive oxygen species, antioxidative enzymes.

Introduction

The role of copper in plants depends greatly on its concentration. Copper in trace amounts is an essential micronutrient for algae and higher plants for its role as a cofactor for metabolic processes like photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, ethylene perception, oxidative stress reduction, cell expansion and cell-wall lignification. At higher concentrations, copper can induce several negative effects including generation of reactive oxygen species, exchange of essential metal ions from the active sites and visible symptoms such as chlorosis, necrosis and growth inhibition (Marschner, 1995; Prasad, 2004). A well coordinated procedure of uptake, buffering, translocation and storage processes is necessary to uphold essential concentrations of the metal in various tissues and compartments within the narrow physiological limits (Clemens *et al.,* 2002). Copper is transported into the plant cell by COPT family of transporters on the plasma membrane which has been described as a group of highly hydrophobic proteins; all its members contain 3 trans-membrane domains

and specific Cu^{2+} binding site rich in methionine and histidine residues at the amino terminus (Kampfenkel *et al.,* 1995; Sancenon *et al.,* 2003; Andres-Colas *et al.,* 2006). Copper homeostasis is maintained inside the cell by copper chaperones which sequester copper to a non reactive form and also interact with other transport proteins for delivering copper to its necessary destinations (Himelblau and Amasino, 2000; Company and Gonzalez-Bosch, 2003; Chu *et al.,* 2005). Two P-type ATPases, PAA1 and PAA2, are required for efficient copper delivery across the plastid envelope and the thylakoid membrane, respectively, in *Arabidopsis* (Shikanai *et al.,* 2003; Abdel-Ghany *et al.,* 2005). Inside the root, Cu^{2+} is said to be strongly accumulated in the cortex and the concentration decreases sharply from the outer to the inner cell layers (Adruini *et al.,* 1996; Ducic and Polle, 2005). Copper is poorly translocated by xylem and thus uptake by shoots is very low (Liao *et al.,* 2000).

The aim of this review is to summarize the toxic effects of Cu^{2+} and focus on the recent developments on the various underlying metabolic changes that bring about such toxic effects. We also focus on tea, which is the most popular drink in the world after water. Tea (*Camella sinensis* (L.) O. Kuntze) is a perennial evergreen plantation

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crop with productivity round the year. The harvest includes tender shoots that are plucked normally at one to three weeks interval. This induces further vegetative growth and ensures continuous supply of green flushes (Burgess and Carr, 1997; Karmakar and Banerjee, 2005). Fungal pathogens such as *Exobasidium vexans* are capable of infecting the pluckable tender leaves thereby warranting a regular spraying of copper fungicides in heavy doses especially during the six month long monsoon period (May-October) when fungal infections assume massive proportions. This causes a buildup of Cu^{2+} in the soil over the years and the concentration of Cu^{2+} can easily overcome the threshold limit for toxicity.

Copper in plants

One of the major sites of copper accumulation in plants is the chloroplast. This metal is directly involved as a component of plastocyanin (PC) in the photosynthetic electron transport chain. PC is one of the most abundant proteins of thylakoid lumen (Kieselbach *et al.,* 1998) and is essential for electron transfer between the cytochrome b6f complex and photosystem 1 (Weigel *et al.,* 2003). The metal has a distinct regulatory role in electron transport between the photosystems as the constituent of PC (Maksymiec, 1997). In the chloroplast stroma, Cu/Zn superoxide dismutase (SOD) requires Cu^{2+}, along with Zn, as cofactors to catalyze the dismutation of superoxide radicals (O_2^-) threby forming H_2O_2 and O_2^-. In *Arabidopsis thaliana*, out of seven identified SOD genes, the most active CSD1 and CSD2 genes both encode a Cu/Zn SOD with CSD1 activity in the cytosol and CSD2 activity in the stroma (Kliebenstein *et al.,* 1998). Polyphenol oxidase is another Cu^{2+} protein found in the thylakoids of some plants, such as spinach (Kieselbach *et al.,* 1998), but not in other species such as *A. thaliana* (Schubert *et al.,* 2002). The enzyme has been proposed to be involved in the photoreduction of O_2 by PS I (Vaughn *et al.,* 1988). Cu^{2+} mediates the activity of several other enzymes such as ascorbate oxidase which catalyses the reduction of O_2 to water. The enzyme contains 8 Cu^{2+} ions which participate in the transfer of electrons in presence of ascorbate, the reducing substrate (Maksymiec, 1997). Other important Cu containing proteins within plant cells include the mitochondrial cytochrome-C oxidase enzyme, the ethylene receptors in the endomembrane system and various apoplastic oxidases (Cohu and Pilon, 2007). Copper is also necessary for amine oxidase function where it catalyses oxidative deamination of polyamines with the simultaneous formation of aldehyde, ammonia and H_2O_2 (Maksymiec, 1997).

Copper as a toxic element

Inspite of the indispensability of copper in plant metabolism, excess copper has strong toxic effects. Copper can be limiting to plant productivity in crops when below 5 µg g^{-1} dry weight (DW), whereas toxicity is reported above 30 µg g^{-1} DW (Marschner, 1995). The most common feature of copper toxicity is the decrease in mass of roots. Copper toxicity can be damaging to plant roots, with symptoms ranging from disruption of the root cuticle and reduced root hair proliferation, to severe deformation of root structure (Sheldon and Menzies, 2005; Lequex *et al.,* 2010). Cu^{2+} is toxic to plant cell which lead to plant retardation and leaf chlorosis (Rhoads *et al.,* 1989; Yadav, 2010). High Cu^{2+} concentrations predisposes photosystem II to photoinhibition (Patsikka *et al.,* 2002), causes reduction in chlorophyll content arising from partial destruction of grana and modification of the protein-lipid composition of thylakoid membranes (Lidon and Henriques, 1991; Maksymiec, 1997). Copper toxicity can also results in significant alteration in the concentration of minerals such as Fe, Mg, Ca, Zn, K and Na in both root and shoot (Lidon and Henriques, 1993; Lequex *et al.,* 2010).

Copper is relatively abundant in the earth's crust and better soluble, therefore more mobile than other heavy metals in the surface environment (Flemming and Trevors, 1989). Copper concentration in non-polluted soils range from 10 to 80 ppm Cu^{2+} but soils located near mining areas or metal-processing industries may be contaminated by very large amounts of Cu^{2+} (Hagemeyer, 2004). The bioavailability is determined by the form taken by the metal (ionic, complex

or precipitated) which depends on environmental factors and therefore, varies widely, giving rise to possible conditions of toxicity (Flemming and Trevors, 1989; Greger, 2004). The level of bioavailable copper is increased by human activities which either increases the abundance or causes changes in soil chemistry thus affecting the solubility (Rhoads *et al.,* 1989; Flemming and Trevors, 1989). In the soil, copper remains immobilized onto the organic materials such as fulvic and humic acids and to clay and mineral surfaces. The bioavailibility in soil is strongly dependent on factors such as pH, cation exchange capacity (CEC), clay content, water hardness and organic matter content (Flemming and Trevors, 1989; Greger, 2004; Rooney *et al.,* 2006). Low pH increases the metal availability since the hydrogen ion has a higher affinity for negative charges on the colloids, thus competing with the metal ions of these sites, therefore releasing metals (Greger, 2004). Rhoads *et al.* (1989) found that growth of tomato plants was reduced at soil pH below 6.5 with soil-copper levels above 150 mg. Thus soil properties have a significant impact in the expression of toxicity of copper in plants.

According to Brun *et al.* (2001) agricultural soil in many parts of the world are contaminated by heavy metals. The use of Bordeaux mixture for almost one century against vine downy mildew has caused severe copper contamination of soil in many wine-producing regions (Van-Zwieten *et al.,* 2004). Copper contamination also caused serious problems in cereals such as rice (Lidon and Henriquesa, 1993), wheat (Lanaras *et al.* 1993) and barley (Vassilev *et al.,* 2003). Graham *et al.* (1986) found that excess fungicidal copper reduced seedling growth in citrus and also inhibited colonization of the roots by mycorrhizal fungus. In citrus orchards, stunted tress were produced with less mycorrhizal colonization under higher Cu concentrations and low pH (<5) conditions of the soil. In India, the major tea cultivation area comprises the eastern sub-Himalayan region where the soil is mainly acidic in nature (pH 4.2-5.8) (Singh and Singh 2006). While this is good for tea cultivation (Sarkar, 1994), but it increases the possibility of Cu^{2+}

ions accumulated in the tea garden soils to become more available for absorption by plants which may lead to toxicity.

Copper in tea gardens

An example of an industry in India which depends primarily on copper fungicides is the tea industry. India is second only to China in tea production and the largest consumer of tea in the world. Currently, India produces 23% of total world production. It is the second largest industry in terms of employment and generally drives the economies of the regions where the tea gardens are concentrated, for example Assam and sub-Himalayan West Bengal (Selvakumar and Jeyaselvam, 2012). Tea plants are cultivated extensively as large plantations where it is often allowed to grow under variant soil and climatic condition thereby making them prone to attacks by fungal pathogens. Major diseases include blister blight, brown blight, grey blight and black rot in leaves, and branch canker, thorny blight and pink disease in stems. To control the diseases, copper based fungicides are used excessively in tea gardens of North East India including Assam and sub-Himalayan West Bengal (Barua, 1988). The fungicides that are used most commonly include basic copper sulphate, Bordeaux mixture (a combination of hydrated lime and copper sulphate), Bicoxy (a new formulation of copper oxychloride 50% WP) and various customized formulations of copper sulphate and copper oxychloride (Worthing, 1983; Singh, 2005). A survey covering several tea gardens of the Darjeeling and adjoining Jalpaiguri district of sub-Himalayan West Bengal conducted by the authors has revealed that copper-fungicides are extensively used in the tea gardens of the Dooars and Terai region and also in the hilly regions of West Bengal. Copper based fungicides are used in large scale because they have multisite activity with a low risk of pathogens developing resistance (Van-Zwieten *et al.,* 2004) and are relatively less phytotoxic than Ni based fungicides. In fact, copper based fungicides are highly recommended in literature and are often regarded as the most efficacious and

economic fungicide for controlling the foliar diseases of tea (Singh, 2005).

Mechanisms of Cu²⁺ toxicity

Copper is a redox active metal with an electrochemical potential of -260V. The redox nature of Cu^{2+} ions makes it very useful as a cofactor in electron transfer reactions (Ducic and Polle, 2005). However, the reversible oxidation–reduction property of Cu^{2+} could also result in oxidative stress if Cu^{2+} would be present as a free ion. Heavy metals in general have been recognised as a major toxicant in plant cells due to their capability of generating reactive oxygen species (ROS) such as hydroxyl radical (OH•) superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which can damage the bio-molecules such as membrane lipids, proteins and nucleic acids. During the reduction of oxygen to water, ROS may be produced by a chain of reactions which initially needs energy input but subsequently occur spontaneously. O_2^- is a short-lived and moderately reactive ROS which reduces quinines and transition metal complexes of Fe^{3+} and Cu^{2+} thereby affecting the metal containing transporters and enzymes. O_2^- can additionally combine with protons in aqueous medium and form hydroperoxyl radicals ($HO_2•$) which can induce lipid auto-oxidation in membranes (Shaw et al., 2004). H_2O_2 is relatively long-lived and moderately reactive which oxidises the thiol groups of some enzymes (e.g. enzymes of the Calvin cycle and Cu-Zn SOD) and inactivates them (Vranova et al., 2002). However, the most reactive of all the ROS is the hydroxyl radical (OH•) which can potentially react with all types of biomolecules and in excess can cause cell death because cells do not have any enzymatic antioxidant system to quench it. The radical is formed from H_2O_2 by the Haber Weiss and Fenton reactions and Cu^{2+} being a redox active metal catalyzes the formation of this most harmful active radical (Arora et al,. 2002; Vranova et al., 2002) as summarized below:

$$H_2O_2 + O_2^- \xrightarrow{Cu+, Cu2+} OH^- + OH• + O_2^-$$

Haber Weiss reaction
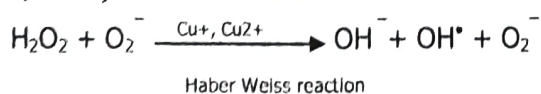
One of the richest sources of ROS in plants is the chloroplast. These can be formed due to the highly energetic electron transfer reactions triggered by chlorophyll excitation along with an excess supply of oxygen. Singlet oxygen ($^1O_2$) can formed during de-excitation of chlorophyll which causes major oxidative damage to biomolecules. High light intensity can cause over reduction of PS I and generation of excessive NADPH which cannot be utilized by the CO_2 fixation process thereby reducing the NADP⁺ pools. O_2 which is abundant in the chloroplast can take up electrons from PS I in such a situation, which leads to production of ROS through the Mehler reaction (Sharma et al., 2012). Under conditions of low CO_2 fixation such as cold temperature or low CO_2 availability, excess reduction of an increase in ROS levels can occur even at moderate light intensities. As H_2O_2 or O_2 are only moderately reactive, therefore, the main responsible factor for the intense biological damage is the metal ion which catalyzes the formation of the highly toxic hydroxyl free radical (OH•) from H_2O_2 (Maksymiec, 1997). Thus ROS may be generated in the plant due to several abiotic as well as biotic causes but true damage is caused by the additional metal toxicity.

The hydroxyl radical (OH•) can either add onto the biological molecules or eliminate hydrogen from them by forming water. The hydroxylated biomolecules can in turn hydroxylate other molecules, thereby, initiating a chain of reaction or change to stable oxidised products. The activated hydroxylated molecules can also dismute themselves by forming intermolecular cross links (Shaw et al., 2004). Oxidised Cu^{2+} ions can be actively involved in electron transfer during formation of stable oxidized products. In reactions where the OH• radical eliminates H from biomolecules, it leaves an unpaired electron in the organic molecule thereby forming a reactive organic radical which can then react with oxygen to form peroxy radical (ROO•). The peroxy radical is again a reactive species and can eliminate hydrogen from other biomolecules and change them into organic radical products thereby creating a chain of reactions. The peroxidation reaction is evident in lipid peroxidation reactions that take place in cell membranes to form lipid peroxides (ROOH) (Shaw et al., 2004; Arora

et al., 2002). However, in presence of reduced Cu^{2+} ions which can participate in Fenton reaction (shown below), the highly reactive alkoxy radical (RO^-) is formed from the ROOH which is as damaging as the hydroxyl radical thus opening up another cascade of immensely damaging oxidative reactions.

$$ROOH + Cu^+ \longrightarrow OH^- + Cu^{2+} + RO^-$$

A study on the toxicity mechanisms suggest that the generation of reactive oxygen species is a natural phenomenon but is increased to alarming proportions due to presence of stress factors. Presence of Cu^{2+} ions above the threshold limit is immensely stressful to plants due to its redox nature as it can catalyze and enhance the formation of all types of ROS by participating actively in several types of oxidative reactions.

Plant response to Copper toxicity

Plants have developed a wide range of protective mechanisms for mitigating copper toxicity. Primary defence mechanisms prevent metal to enter into the cell via exclusion, or binding of metal to cell wall and other ligands, organic acids, amino acids, glutathione (GSH) or phytochelatins (PCs) to render them harmless (Antosiewicz and Wierzbicka, 1999). Antioxidative mechanisms that control the level of ROS and shield the system before the sensitive parts of the cellular machinery gets damaged are mediated by molecules which have been broadly divided into two types, the high molecular weight enzymatic catalysts and the low molecular weight antioxidants (Pinto et al., 2003). The enzymes involved in scavenging ROS include SOD, catalase (CAT), peroxidases (POD) and glutathione peroxidase and those involved in detoxifying lipid peroxidation products include glutathione-S-transferases (GST), phospholipid-hydroperoxide glutathione peroxidase and ascorbate peroxidase (APX). Table 1 enlists the different enzymes which have been studied in relation to copper toxicity. The low molecular weight compounds that act as cellular antioxidants are ascorbate, glutathione, phenolics, flavonoids, carotenoids and tocopherols.

Besides these, a whole array of enzymes is needed for the regeneration of active forms of the antioxidants such as monohydroascorbate reductase and glutathione reductase (Blokhina et al., 2003; Pinto et al., 2003).

Binding of copper and its sequestration

Plant adapt to heavy metal stress by acquiring several strategies, the most prominent being the synthesis of phytochelatins and metallothioneins which contribute to metal detoxification by chelation of the metal ions. Phytochelatins are simple thiol rich metal binding peptides containing glutamate, cystein and glycine in ratios of 2:2:1 to 11:11:1 (Grill et al., 1985; Prasad, 2004). These peptides are synthesized non-translationally from glutathione in the presence of heavy metals by the enzyme phytochelatin synthase (Grill et al., 1989). Apart from being a precursor to phytochelatins, glutathione is also an important antioxidant molecule, which plays a predominant role in protection against free radicals (Alscher, 1989). Copper induced increase in phytochelatin synthesis results in oxidative stress through the depletion of the antioxidant glutathione. De Vos et al. (1992) showed that copper tolerance in the plant species *Silene cucubalus* does not depend on the production of phytochelatins but is related to the ability of this plant to prevent glutathione depletion resulting from copper-induced phytochelatin production.

Metallothioneins are low molecular weight proteins with high cystein content, which bind metal ions to form metal thiolates and metal thiolate clusters. Class III metallothioneins are found in plants and is reported to be induced by the presence of a variety of metals including Cd, Cu, Zn, Pb, Hg and Ag (Hamer, 1986; Prasad, 2004). However, phytochelatins rather than metallothioneins are mainly responsible for detoxification of toxic heavy metals (Yadav, 2010). Moreover, metal binding ability is higher in phytochelatins than in metallothioneins on a per-cysteine basis (Mehra and Mulchandani, 1995). In addition, phytochelatins possess the ability to scavenge

Table 1. Enzymes/Metabolites whose levels have been studied after copper exposure

Enzyme/Metabolite	Plant	Location	Reference
Peroxidase	*Zinnia elegans* and *Cosmos sulfureus*	Shoots and roots	Tsay *et al.* 1995
	Zea mays	Leaves and roots	Mocquot 1996
	Helianthus annuus	Leaves and roots	Garcia *et al.* 1999
	Oryza sativa	Leaves	Fang and Kao, 2000
	Capsicum annum	seedlings	Diaz *et al.* 2001
	Phaseolus vulgaris	Leaves and roots	Cuypers *et al.* 2002
	Allium sativum	Leaves and roots	Meng *et al.* 2007
	Erica andevalensis	Leaves, Roots	Oliva *et al.* 2010
	Zea mays	Roots	Zhao et al 2010
	Vigna mungo	seedlings	Solanki *et al.* 2011
	Beta vulgaris	leaves	Morales *et al.* 2012
	Camellia sinensis	Leaves	Saha *et al.* 2012
Catalase	*Avena sativa*	Leaves	Luna *et al.* 1994
	Lycopersicon esculentum	Leaves, stem and roots	Mazhoudi *et al.* 1997
	Oryza sativa	seedlings	Chen *et al.* 2000
	Camellia sinensis	root	Ghanati *et al.* 2005
	Prunus cerasifera	seedlings	Lombardi and Sebastiani, 2005
	Zea mays	roots and shoots	Pourakbar *et al.* 2007
	Vigna mungo	seedlings	Solanki *et al.* 2011
	Atriplex halimus	leaves	Brahim and Muhamed, 2011
	Cucumi sativus	Roots	Iseri *et al.* 2011
Superoxide dismutase	*Nicotiana tabacum*	leaves	Pitcher *et al.* 1991
	Glycine max	root	Chongpraditnun *et al.* 1992
	Nicotiana tabacum and *Pisum sativum*	leaves	Sen Gupta *et al.* 1993 Hartley-Whitaker *et al.* 2001
	Holcus lanatus	Root	Wang *et al.* 2004
	Brassica juncea	Roots	Ghanati *et al.* 2005
	Camellia sinensis	Root	Lombardi and Sebastiani, 2005
	Prunus cerasifera	Root and shoot	Peng *et al.* 2006
	Elsholtzia splendens	Root, stem and leaves	Ke *et al.* 2007
	Daucus carota	Roots and leaves	Meng *et al.* 2007
	Allium sativum	Leaves and roots	Zhang *et al.* 2008
	Elsholtzia haichowensis	Root	Gao *et al.* 2008
	Jatropha curcas	Root, stem and leaves	Tie *et al.* 2012
	Zea mays	Leaves	Azooz *et al.* 2012
	Triticum aestivum cv. Hasaawi	seedlings	
Ascorbate peroxidase	*Avena sativa*	Leaves	Luna *et al.* 1994
	Lycopersicon esculentum	Leaves, stem and roots	Mazhoudi *et al.* 1997
	Phaseolus vulgaris	Leaves and roots	Weckx and Clijsters, 1996
	Oryza sativa	root	Chen *et al.* 2000
	Camellia sinensis	Root	Ghanati *et al.* 2005
	Morus rubra	Leaves	Tewari *et al.* 2006
	Oryza sativa	Root and shoot	Thounaojam *et al.* 2012
	Camellia sinensis	Root and shoot	Hajiboland and Bastani, 2012
	Camellia sinensis	Leaves	Saha *et al.* 2012
γ-glutamylcysteinyl synthetase	*Camellia sinensis*	Leaves	Yadav and Mohanpuria, 2009
	Triticum aestivum	Leaves	Shan *et al.* 2012
Glutathione reductase	*Silene cucubalus*	root	De Vos *et al.* 1992
	Panax ginseng	Roots	Ali et al. 2006

	Morus rubra	Leaves	Tewari *et al.* 2006
	Zea mays	Roots and leaves	Pourakbar *et al.* 2007
	Oryza sativa	Root and shoot	Thounaojam *et al.* 2012
	Triticum aestivum	Leaves	Shan *et al.* 2012
	Zea mays	Roots	Wang *et al.* 2011
	Zea mays	Leaves	Tie *et al.* 2012
Dehydroascorbate reductase	*Cucumis sativus*	Roots and leaves	Arora *et al.* 2002
	Panax ginseng	roots	Ali *et al.* 2006
	Triticum aestivum	Leaves	Shan *et al.* 2012
Phenylalanine ammonia lyase	*Phyllanthus tenellus*	Leaves	Santiago *et al.* 2000
	Camellia sinensis	leaves	Basak *et al.* 2001
	Camellia sinensis	leaves	Chakraborty *et al.* 2002
	Matricaria recutita	Root and leaves	Kovacik and Backor, 2007
	Glycine max	roots	Chmielowska *et al.* 2008
	Jatropha curcas	Root, stem and leaves	Gao *et al.* 2008
Polyphenol oxidase	*Camellia sinensis*	Leaves	Basak *et al.* 2001
	Jatropha curcas	Root, stem and leaves	Gao *et al.* 2008

ROS and thereby aid in mitigating oxidative stress (Tsuji *et al.*, 2002).

Accumulation of amino acids like proline has been observed in response to several biotic and abiotic stresses in plants. Content of free proline has been found to be related to Cu^{2+} tolerance in plants (Backor *et al.*, 2003; Chen *et al.*, 2004). Excess Cu^{2+} has been found to result in inadequate proline (Thomas *et al.*, 1998) and lead to the malfunctioning of copper exclusion machinery (Chen *et al.*, 2004). Copper complexes with amino acids such as proline, histidine or nicotinamine play important role in xylem sap transport (Liao *et al.*, 2000).

Antioxidant response

Plants possess well developed defence system against ROS which restricts its formation and maneuver its removal. Inside the plant cell, superoxide dismutases (SOD) provide the first line of defence against ROS. The enzyme is located in different cell compartments including mitochondria, chloroplast, glyoxisomes, peroxisomes, microsomes, apoplast and cytosol (Alscher *et al.*, 2002) and catalyzes the disproportionation of O_2 to H_2O_2 and molecular oxygen (Scandalios, 1993). SOD enzymes are classified based on the metal cofactors: the Cu- Zn SOD, the Mn-SOD and Fe-SOD (Bowler *et al.* 1994). Although each type of SOD predominates in specific cell compartments, their occurrences are not restricted, and all types can be detected in most of the cellular locations (Arora *et al.*, 2002). An increased level of SOD has been correlated to enhanced oxidative stress protection in plants (Sen Gupta *et al.*, 1993). Increase in SOD activity has been reported against copper induced stress in tolerant plants such as *Prunus cerasifera* (Lombardi and Sebastiani, 2005); *Elsholtzia haichowensis* (Zhang *et al.*, 2008); *Elsholtzia splendens* (Peng *et al.*, 2006); *Jatropha curcas* (Gao *et al.*, 2008); *Holcus lanatus* (Hartley-Whitaker *et al.*, 2001); *Daucus carota* (Ke *et al.*, 2007); *Ceratophyllum demersum* (Rama Devi and Prasad, 1998); *Brassica juncea* (Wang *et al.*, 2004); *Hydrilla verticillata* (Srivastava *et al.*, 2006); *Zea mays* (Tie *et al.*, 2012), *Triticum aestivum* cv. Hasaawi (Azooz *et al.*, 2012), *Allium sativum* (Meng *et al.*, 2007) etc. However, Weckx and Clijsters (1996) observed that SOD was not involved in the defence mechanism against copper induced oxidative stress in primary leaves of *Phaseolus vulgaris*. Contradictory results have also been recorded regarding the response of catalase (CAT) against copper stress. Both CAT and peroxidase (POD) are involved in the removal of H_2O_2 that accumulates due to dismutation of O_2 by SOD. Catalase activity did not increase in Cu^{2+} stressed roots of rice seedlings (Chen *et al.*, 2000) or in black gram (*Vigna mungo*) seedlings (Solanki *et al.*, 2011). On the other hand, CAT activity was reported to increase in

A. halimus leaves (Brahim and Muhamed, 2011) *Prunus cerasifera* (Lombardi and Sebastiani, 2005), *C. sativus* roots (Iseri *et al.*, 2011) and in maize roots and shoots (Pourakbar *et al.*, 2007) in response to excess Cu^{2+} concentrations. The mobilization of POD in response to Cu^{2+} induced oxidative stress in plants is well accepted (Fang and Kao, 2000; Diaz *et al.*, 2001; Cuypers *et al.*, 2002; Meng *et al.*, 2007; Solanki *et al.*, 2011). Apart from POD and CAT, the enzymes and metabolites of the ascorbate-glutathione cycle are also involved in the removal of H_2O_2. The majority of these enzymes [ascorbate peroxidase (APX), glutathione reductase (GR), and dehydroascorbate reductase (DHAR)] have been found in chloroplasts, cytosol, mitochondria, and peroxisomes (Dat *et al.*, 2000). Glutathione and ascorbate accumulate in these cellular compartments and their redox state is maintained through glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR). All these enzymes along with ascorbate and glutathione have a pivotal role in defence against ROS induced oxidative damage (Arora *et al.*, 2002; Yruela, 2005; Sharma and Dietz, 2008; Shan *et al.*, 2012). De Vos *et al.*, (1992) observed that glutathione depletion is the major cause of Cu^{2+} induced oxidative damage in Cu^{2+} sensitive *Silene cucubalus* plants. It has been shown that tolerance to a copper-enriched environment, and the accompanying oxidative stress in *Enteromorpha compressa* occurs through the accumulation of copper, activation of ascorbate peroxidase, synthesis of ascorbate (accumulated as dehydroascorbate) and consumption of glutathione and water-soluble phenolic compounds (Ratkevicius *et al.*, 2003).

Stress in tea

A literature survey revealed that several studies have been conducted on different types of abiotic stresses in tea. Plants of different cultivars of tea have been grouped into the tolerance classes: susceptible and resistant, in response to drought stress (Chakraborty *et al.*, 2002; Damayanti *et al.*, 2010), cold stress (Upadhyay, 2012) and heavy metal stress (Yadav and Mohanpuria, 2009). Several parameters have been identified such as rates of photosynthesis and transpiration, relative water content, stomatal conductance and leaf total soluble sugar content (Damayanti *et al.*, 2010), root and shoot extension (Burgess and Carr, 1997), levels of proline and antioxidative enzymes (Chakraborty *et al.*, 2002; Upadhyay and Panda, 2004; Upadhyay *et al.*, 2008), morphological characters (Waheed *et al.*, 2012) etc. in order to screen tea cultivars for drought tolerance. Additionally, studies on alterations in bioconstituents that determined quality of tea in the tea clones under soil moisture revealed a decrease in PAL activity in both tolerant and susceptible clones which correlated with a lower flavonol content and quality deterioration (Jeyaramaja *et al.*, 2003).

Tea plants exposed to excess heavy metals have shown several alterations in physiological and biochemical parameters. Increased level of lipid peroxidation and a reduction in photosynthetic rate, transpiration rate, chlorophyll and protein content and biomass production were found in plants exposed to excess Cd (Mohanpuria *et al.*, 2007; Shi *et al.*, 2008). Oxidative stress was evident as the transcript levels of glutathione biosynthetic genes showed up-regulation while glutathione-S-transferase (GST), the enzyme which help in sequestration of high levels of metal ions to vacuole, did not show any change on Cd exposure (Mohanpuria *et al.*, 2007). Hajiboland and Bastani (2012) observed that CO_2 assimilation and dry matter production decreased while antioxidant enzyme activity and proline content increased significantly in tea plants under Boron deficiency and water stress. Mukhopadhyay *et al.* (2013) observed that both deficiency and excess in zinc caused a considerable decrease in shoot and root fresh and dry masses. Zinc stress decreased net photosynthetic rate, transpiration rate, stomatal conductance, and content of chlorophylls *a* and *b and increased the content of* superoxide anion, malondialdehyde, hydrogen peroxide, and phenols. Although the activities of ascorbate peroxidase, catalase, superoxide dismutase, and peroxidase as well as expression of respective genes were up-regulated, the authors concluded that the overall antioxidant

system did not afford sufficient protection against oxidative damage (Mukhopadhyay *et al.,* 2013). Treatment of tea plants with excess heavy metals such as mercury (II) and nickel (II) decreased the chlorophyll content of the leaves, along with a significant reduction in Hill activity (Basak *et al.,* 2001). The activities of antioxidative enzymes viz. Superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) was increased by Aluminium in the roots of cultured tea cells and also in intact plants (Ghanati *et al.,* 2005). Aluminum (Al) inhibited tea pollen tube growth but the effect was found to be alleviated by fluorine (Konishi and Miyamoto, 1983) which is accumulated by tea plants normally in high excess (Ruan *et al.,* 2004). Tea plants tolerated fluorine at concentrations < 0.32 mM (Li *et al.,* 2011). Fresh and dry mass, chlorophyll content and net photosynthetic rate decreased while proline, malondialdehyde and hydrogen peroxide contents increased with increasing fluorine concentrations. Activity of antioxidant enzymes also showed significant alterations thereby suggesting that antioxidant defence system of leaves did not sufficiently scavenge excessive reactive oxygen species generated due to excess fluorine (Li *et al.,* 2011).

Cu^{2+} stress in tea

Although copper based fungicides are being used in tea gardens for several decades (Sarmah, 1960), we know little about the role of excess Cu^{2+} on tea plants and at what concentrations it may be considered as a pervasive threat (Saha *et al.,* 2012). Only a few studies have focused on Cu^{2+} toxicity in tea (Basak *et al.,* 2001; Yadav and Mohanpuria, 2009; Saha *et al.,* 2012) and these have revealed that number physiochemical parameters are altered on exposure to excess copper. For example, the chlorophyll and protein contents were found to decrease in Cu^{2+} treated plants (Basak *et al.,* 2001; Yadav and Mohanpuria, 2009; Saha *et al.,* 2012). Germination of tea seeds were also affected in presence of excess copper. Substantial reduction in the length and biomass of root and shoot was observed (Mandal et al., 2013). Excess Cu^{2+} caused an increase in lipid peroxidation, phenolics and

antioxidative enzyme levels such as POD, SOD and APX in multiple cultivars of tea (Saha *et al.,* 2012). A significant difference among cultivars was noted where the more sensitive cultivar seemed to lose its antioxidative capacity at Cu^{2+} concentrations higher than 400 µM while the more tolerant cultivar was able to withstand a maximum of 600 µM of Cu^{2+} ions. Two new isozymes were also found to be induced in the leaves of tea exposed to high concentration of Cu^{2+} (Saha *et al.,* 2012). Yadav and Mohanpuria (2009) observed that expression of the enzymes γ-glutamylcysteinyl synthetase, glutathione synthetase and phytochelatin synthase was elevated more in the tolerant tea cultivar than the susceptible one when exposed to excess Copper and Aluminium.

Conclusion

Heavy metal stress is one of the major problems that limit agricultural productivity of plants. Plants show relative differences in their heavy metal tolerance capacity among the species and also among cultivars of the same species. Copper stress in general induces ROS and generates oxidative stress. It has been found that in addition to accumulated metal ions, high levels of ROS adversely affected the plants. Such ROS related damages have been observed in tea cultivars also. Although of the negative impact of excess Cu^{2+} in tea plants have been documented, the level of Cu^{2+} accumulation caused due to long term application of Cu^{2+}-based fungicides in tea gardens and its bioavailability under tea garden conditions are yet to be studied. Additionally, more detailed studies on mechanisms of Cu^{2+} toxicity in the tea plant, especially at the gene level are necessary. Identification of genetic determiners of tolerance may make the resistant cultivars a potential source for genetic manipulation of other important elite cultivars.

Acknowledgement

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Review Article
Rice research in the high-throughput sequencing era: Genomic breeding
Rice breeding for better health

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Abstract

Rice [*Oryza sativa* L.] is the most important cereal crop belongs to the family Poaceae (Grass) which provide staple food for half of the World's population (>3.3 billion). This staple food grain (rice) supplies the main energy resource providing 40-75% of the daily calorie intake to the world's poor people. It is equivalent to the proposition that 'Rice is life' in Asian continent because 90% people dependent on for their sustainable livelihood. Simultaneously Asia is considered as 'Rice Basket' because it produces 90% of the world's production (662 million tons, paddy rice, Mt). Total world production was 729 Mt from 154.3 million hectares with productivity of 4.1 tons/hectare (t/ha) in 2012 of which 662 million tons produced by Asian countries. Rice production has been doubled in the recent decades (1960s-1990s) during the time of Green Revolution (1960s) primarily as the result of genetic improvement. It was factual that the varieties released in the last 30 years in the farmers field, had a narrow genetic base in-spite of high genetic diversity prevailed in the rice germplasm, and yield enhancing capacity has reached to plateau. We need more production of rice to feed 9 Billion people in 2050. Breeder could manage the yield increase over released varieties through genetic gain by combining the yield related genes/QTLs from various genetic resources of rice germplasms either from cultivated local landraces or from wild varieties. Germplasm diversity is the mainstay for crop improvement and genetic dissection of complex traits. Rice germplasm shows tremendous genetic diversity in both within the species and among the varietal groups. This genetic diversity may be associated with the diverse alleles of important traits and can be exploited to introgress these traits using knowledge of molecular breeding techniques such as marker assisted breeding (MAB) or marker assisted selection (MAS). The Next Generation Sequencing based technology is used for whole genome analysis to unveil the genetic and genomic information pertaining to important traits for advancing the molecular breeding procedures to increase the production. That ultimately leads to the development of genomic breeding and genomic selection to accelerate the breeding process.

Key words: Rice, Molecular Breeding, Genomic selection, MABC breeding, MAS, GAB.

Introduction

Genomic Breeding [Genomic Assisted Breeding (GAB) and Genomic Selection (GS)]
Rice genome sequencing information has revolutionized the research dimension in rice genetics and breeding and as a result many varieties were improved that will continue to feed the growing world population (Scott, 2016). Now, rice has been considered as a model crop for genetics and breeding. Genome sequencing information of rice allows the breeders to better understand the genetic variation and can exploit this genetic variation to improve the HYV (McCouch *et al.*, 2012). Molecular understanding of the genetic basis of traits related to N and P-use has been acquired from the genome sequencing analysis and that has been utilized in engineering 'Green Super Rice' (GSR) to enhance the yield and quality of rice with minimum inputs (Zhang, 2007). Marker assisted selection (MAS) is an indirect process of selection where screening and individual plant identification is carried out on the basis of markers (DNA markers) instead of the expressed phenotypic trait. Therefore, thriving benefits of MAS depends on the tight association between the markers and the gene (or *QTLs*) responsible for that traits. Recombination associated phenomenon between linked markers and the gene(s) limits the applicability of MAS breeding. The application of intragenic markers, termed as functional markers can help to surmount this

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problem (Andersen and Lübberstedt, 2003). Thus, new type of genomic tools such as NGS can accelerate the identification of markers tightly linked to target genomic regions. Limitations of MAS procedure can be overcome using a discrete system known as genomic selection (GS or genome-wide selection), based on the high-throughput Next Generation Sequencing (NGS) technique. Huge amount of genomic information can be assembled using the Next Generation Sequencing (NGS) technologies, which allows mass sequencing of genomes and transcriptomes. The bioinformatics analysis of this genomic data escorts to the development of large collections of molecular markers leading to the discovery of new gene/*QTLs*, position and dissection of complex traits. The NGS technology has revolutionized the genotyping ability (gene and *QTLs* discovery) of large population that help breeders to accelerate the breeding program. The SNPs markers are available in this NGS era and thus becoming the preferred choice of marker system in modern genomics research (Ganal *et al.* 2009). SNPs are more abundant, stable, amenable to automation, and most cost-effective in this NGS system. Genes and *QTLs* are identified through genome-wide association studies (GWAS) based on association mapping which also termed as linkage disequilibrium (LD) mapping (phenotype-genotype associations). High-density genome markers are being successfully used in background and foreground selection (also termed as positive selection) in rice breeding. Due to the advantage of the NGS technique, it is now possible to re-sequence the whole genome of any rice varieties to study the genetic diversity and variations. Re-sequencing of genome is constructive in order to the genome-wide discovery of markers for the building of high density genetic maps based on SNPs or SSR markers. These types of SSR/SNP markers are exploited in marker assisted selection (MAS), which include marker assisted backcross selection and helps the breeder to design the genotype of a cross and commonly known as 'Breeding-by-Design', because genotype of the individual is predetermined and predesigned. The same

markers may be utilized in 'genomic selection' (GS) in the genomic breeding technique (Pérez-de-Castro *et al.,* 2012) to improve the rice varieties. Genomic selection (GS) is a new type of breeding method (Genomic breeding) in which genome-wide markers is used to predict the breeding value of individuals in a breeding population.

In this way, many genes and *QTLs* of agronomically valuable traits are identified and information about LD (linkage disequilibrium) and millions of SNPs data are detected from the genome sequencing analyses, which as a whole accelerating the genomic breeding to improve the varieties. The genomic breeding approach can be used as an advanced tool to the alternative of the conventional marker-based genotyping process for discovery of gene (s) and *QTL*. Marker assisted selection (MAS) in molecular breeding system has accelerated the development of crop varieties with improved yield, quality and biotic and abiotic stress tolerance traits. But these traits are complex and governed by many genes, each with small effect. The traditional marker-assisted selection (MAS) has not been so effective to analyse such traits. Marker-assisted selection (MAS) has failed to significantly improve polygenic traits (Bernardo, 2008; Xu and Crouch, 2008). To solve these difficulties, a novel statistical method has been employed to enable the simultaneous estimation of all marker effects and we can get genomic selection (GS) (Meuwissen *et al.,* 2001). The introduction of genomic selection (GS), however, has shifted that paradigm in the era of low cost genome sequencing regime (Jannink *et al.,* 2010). Genomic selection uses a 'training population' of individuals that have been both genotyped and phenotyped to develop a model that takes genotypic data from a 'candidate population' of untested individuals and produces genomic estimated breeding values (GEBVs) (Heffner *et al.,* 2009). In simulation studies, GEBVs based solely on individuals' genotype have been remarkably accurate. Some statistical methods have been used (Best Linear Unbiased Predictors (BLUP)' Bayesian regression, machine learning methods) to develop prediction models for genomic

selection (GS). Both the markers, SSR-like multiallelic markers and SNP-like biallelic markers has given the same prediction value, thus both are reliable in the GS.

Mapping population

In molecular breeding system, mapping populations are mandatory which facilitates *QTL* mapping and gene function analysis based on association studies. Different categories of populations are developed and used as mapping population such as bi-parental and multi-parent mapping populations, mutant populations, and immortalized recombinant inbred lines (RILs), BILs, F2 populations. Primary knowledge is gained from the analyses of mapping population using DNA markers subsequently are being utilized for the identification and map location of agronomically important genes/*QTLs* that provides the basis for marker-assisted selection (MAS) in plant breeding or in genomic selection (GS).

QTL mapping through conventional markers such as SSR, produced low resolution linkage map and time consuming. Most of the agronomically important traits are associated with multiple genes and named as *QTL* (Quantitative Trait Loci). Thus cloning and identification of a gene/*QTL* related to the traits is somewhat problematic and laborious using marker based technique. It is easy if any one uses the sequencing-based genotyping to discover gene or *QTLs* (Huang *et al.,* 2012). Genomic selection is carried out based on simultaneous estimation of effects on phenotype considering all *loci*, haplotype pattern, and markers available to calculate genomic value which ultimately used to select the desired phenotypes. These estimated value is termed as the genome estimated breeding values (GEBVs), are the output from a model of the relationship between the genome-wide markers and phenotypes of the individuals undergoing selection. Varshney *et al.* (2014) studied the need of genomics breeding in crop improvement using the NGS technology which act as highly multiplexed genotyping system and used in many different ways for genotyping the individuals such as whole genome sequencing (WGS), whole genome re-sequencing (WGRS), and genotyping by sequencing (GBS) system. Genome-wide association studies (GWAS) method based on NGS system has offered an effective technique to analyze the genetic architecture of complex traits and allowed identification of candidate genes for further improvement of agronomically important traits (Huang *et al.,* 2010; 2012).

Genomic Era in Rice Breeding

The International Rice Genome Sequencing Project has released the completed genome sequencing report of cultivar Nipponbare of *O. sativa* ssp. japonica (IRGSP 2005) in the year 2005. Before that draft genomic sequence of this Nipponbare cultivar was published in 2002 (Yu *et al.,* 2002). In the same year, the draft genomic sequence of one Chines cultivar 93-11 of subspecies *indica* was also released (Goff *et al.,* 2002). Three rice genome sequences are now available as reference genome such as *Aus* rice cultivar Kasalath, *indica rice* variety 93-11 (Kanamori *et al.,* 2013; Gao *et al.,* 2013) and Nipponbare of *japonica* rice cultivar (IRGSP, 2005). Genomic information acquired from these reports is helpful in rice breeding by analysing the functional genomics markers. Reference genomes are served as a source of gene catalogue to identify high density polymorphic markers, which may be related to the genes of particular traits (Feltus *et al.,* 2004). The Nest Generation Sequencing (NGS) technologies such as Illumina/Solexa (Gao *et al.,* 2012) have revolutionized the genotyping and functional genomics approaches for discovering new genes and alleles to accelerate the breeding process. The availability of high quality whole genome sequence provided a thorough understanding of the genome structure and evolution patterns. Novel means of understanding the genome functions could be designed by utilizing the DNA sequence information. These developments led to the birth of a new discipline in biology aptly termed as "Genomics".

Short reads of the re-sequencing (NGS) data from rice varieties can accurately align using any one as the reference genome from these three. The comparative analysis can also be done using these three reference

genome at a time to analyze genetic variation (Han and Huang, 2013) including SNP, InDel (insertions-deletions), SV (structural variation), and CNV (copy number variations). Based on the reference genome, simple sequence repeat (SSR) markers are now easily available for any region of the rice genome because we now know the each and every base pair of rice genome (such as Nipponbare, indica 93-11, Kasalath). Simple sequence repeat (SSR) markers are easily available for any region of the genome, and candidate gene markers are being developed rapidly. Now, rice is considered as a model plant in the cereal crops. Genome sequencing information of the these reference genome (*indica*, aus, *japonica* rice subspecies) have provided breeders with the necessary tools for marker assisted breeding (MAS).

Application of Marker Assisted Selection (MAS)

In MAS breeding, markers associated with the genes are the main indication for gene mapping and identification. Many genes and *QTLs* with major effects are being utilized to develop improved varieties of rice using marker assisted backcrossing breeding (MABC). Submergence tolerant mega varieties has been development and released within 2-3 years was a significant paradigm shift in rice breeding with MAS. The marker assisted selection (MAS) and marker-assisted back-crossing (MABC) systems have been used in rice breeding to incorporate the genes/*QTLs* from wild or unadapted genetic resources (Septiningsih *et al.,* 2009; Imai *et al.,* 2013; Uga *et al.,* 2013).

The NGS based re-sequencing knowledge is used to identify genome wide variation within a species and genetic diversity within the population and to determine availability of linkage disequilibrium (Huang *et al.,* 2010; Jeong *et al.,* 2013; Guo *et al.,* 2014) among the varieties. Re-sequencing based generated data are applicable in the studies of high-throughput genotyping, and have been used for large-scale gene discovery in rice from RIL lines (Huang *et al.,* 2009), haplotype construction (Xie *et al.* 2010); *QTL* mapping for culm length (Xu *et al.,* 2010), *QTL* detection for

grains (Yu *et al.,* 2011); identifying 49 *QTLs* for 14 agronomic traits and gene discovery in rice (Wang *et al.,* 2011); genome-wide association studies (GWAS) for agronomic traits (Huang *et al.* 2010, 2011); identifying agronomic *QTL* in rice (Xu *et al.,* 2012); and rapid *QTL* mapping in rice (Takagi *et al.,* 2013). Millions of single nucleotide polymorphism (SNP) and insertion-deletion (InDel) markers have been identified in rice based on genome sequencing results.

Thus rice genome has been saturated with such sequence based markers SSR and SNP which is accelerating fine mapping of genes/*QTLs* and developing gene-based allele-specific markers. Markers based genomic information is necessary for the improvement of rice breeding programs. This genetic information are used to study the genetic variation, *QTL* identification (Quantitative Trait Loci) by GWAS, origin of cultivated rice from wild species and help in molecular breeding strategies. Both the processes are employed in background selection integrated with foreground selection to identify the superior lines with maximum recovery of Basmati rice genome with bacterial blight resistance genes (*xa13* and *Xa21* genes) (Gopalakrishnan *et al.,* 2008). Allelic variant can also be detected by using genomic study based on TILLING and EcoTILLING technology, through these approaches one can screen mutant lines and germplasm collections for their genetic variation to detect agronomically important trait specific genes.

Genomic Assisted Breeding (GAB) and Genomic Selection (GS)

Sequencing-based GWAS is employed for the purpose of genetic mapping and uncovering the genetic variability existed among the landraces of rice from the mapping populations (Abe *et al.,* 2012; Huang *et al.,* 2013). Some studies have been performed to identify the allelic variations existing in the rice germplasm through the technique of next generation sequencing (NGS) technologies (Huang *et al.,* 2010; Xu *et al.,* 2011; Huang *et al.,* 2012; 3K RGP, 2014). The NGS based technology is used in re-sequencing the whole genome of rice to unveil the genetic

and genomic information pertaining to important traits for advancing the molecular breeding procedures to increase the production.

The genomics assisted breeding (GAB) is based on genomic selection. In the genomic selection (GS), breeder uses all available markers information within a mapping population to predict genomic estimated breeding values (GEBVs) as a whole. The GAB system has an advantageous value over conventional breeding where genotyping data obtained from a seed or seedling stage can be used to estimate the trait related performance of mature plants without waiting for full grown plants in the fields, which reduces the time and cost for breeding program (Varshney *et al.,* 2005). The MAGIC (multi-parent advanced generation inter-cross) mapping populations are used to shuffle the genetic background among a set of diverse parental lines and increase recombination, which consequently increase resolution of *QTL* mapping (Bandillo *et al.,* 2013). Blast resistance gene *Pii* in rice was identified using WGRS technique, which is a NBS-LRR (nucleotide-binding site-leucine rich repeat) type protein gene (Takagi *et al.,* 2013). Grain yield and yield under drought conditions has been validated in rice using MAS breeding (Imai *et al.,* 2013; Mishra *et al.,* 2013; Venuprasad *et al.,* 2012). Genomics-assisted breeding has radically changed the approach so that breeders can use unadapted genetic resources in breeding program to improve rice varieties. Blast disease is a devastating disease of rice caused by fungal pathogen *Magnaporthae grease.* The resistant *pi21* allele has been identified through NGS approach from japonica rice lines, and can be used to improve blast resistance of rice worldwide without any lingkage drag (Fukuoka *et al.,* 2009).

Genomics-based genotyping system not only reduces the number of breeding cycles but also precisely integrate target genes for particular traits into an ideal genetic background. Twenty-eight genes of important traits were detected in rice using whole genome based SNP array, RICE6K, which is a (Yu *et al.,* 2014). Results also detected 12

SNPs per 1 Mb and provided more intensive information about polymorphisms between *indica* and *japonica* subspecies as well as varieties within *indica* and *japonica* groups. It showed that SNP chip RICE6K is suitable for rice germplasm fingerprinting, functional allele detection, genetic background selection among breeding lines and considered that this genotyping technique can be used reliably in rice genomic breeding. The RICE6K was developed using four million SNPs identified from resequencing results of 500 rice germplasm. Whole genome sequencing results (Huang *et al.,* 2012) demonstrate that *O. sativa* has been domesticated from a single origin of *O. rufipogon.* Many other research reports supporting the view that cultivated rice *Oryza sativa* has been developed from its close wild relatives *O. rufipogon* and *O. nivara* based on genome information (McNally *et al.,* 2009; Zhao *et al.,* 2010, 2011; Molina *et al.,* 2011).

The NGS based GWAS mapping has created a new avenues to accelerate the mining of diverse germplasm to identify important functional alleles (Zhang *et al.,* 2008; Ikeda *et al.,* 2013). Identified functional alleles may help to design new idiotype super rice by combining heterosis vigour between *indica-japonica* (Guo and Ye, 2014) subspecies and it can be used to develop 'Green Super Rice' (GSR) using genomic selection (Zhang, 2007). Rice breeder can utilize the genomic knowledge including DNA sequences and gene functions to create new genotype and control the selection procedure to modify the whole genomic information to improve the varieties through the advent of genomic technologies (*i.e.,* Genomic Breeding). In genomic breeding two types of high-throughput genotyping markers are used, DNA sequencing and DNA array (Davey *et al.,* 2011; Gupta *et al.,* 2008). In genomic breeding, target gene can be selected based on molecular markers and genetic background selection can be achieved using genome-wide DNA polymorphism analyses (Yu *et al.,* 2013). Whole-genome based SNP array (RICE6K) has been used in genomic breeding for fingerprinting the rice germplasm, selection of genetic background

of the progeny lines and target gene introgression (Yu *et al.,* 2013). Using this RICE6K array some functional alleles of seven genes have been detected such as Sd1 (plant height), Gn1a for grain number, grain size gene GW2, plant architecture gene TAC1 and hybrid fertility gene (S5 and Sa) (Yu *et al.,* 2013); rice germplasm characterization based on DNA array (McNally *et al.,* 2009; Wang *et al.,* 2010), GWAS based SNP-genotyping is carried out in rice using 44K SNP array (McCouch *et al.,* 2010; Zhao *et al.,* 2011); Illumina GoldenGate SNP Chip is used in detects SNPs and genetic analysis in rice breeding (Thomson *et al.,* 2012). Whole genome sequencing (WGS) works have been done in many accessions of rice for genetic polymorphism analysis (Arai-Kichise *et al.,* 2014; Lyu *et al.,* 2013; Xu *et al.,* 2012; Yang *et al.,* 2012' Duitama *et al.,* 2015 and 3K RGP). Genetic diversity within *O. sativa* (McNally *et al.,* 2009), integration of important traits in the HYVs (Zhao *et al.,* 2010), and identification of many genes related to complex traits (*QTLs*) has been conducted by the rice scientists using GWAS/SNP chip (Zhao *et al.,* 2011) and aluminium tolerance traits in rice has also been studied in details (Famoso *et al.,* 2011). Yamamoto *et al.* (2012) has developed online data base (OGRO) of rice by incorporating all the functional alleles and *QTLs* to facilities the rice breeding program world-wide. Based on the SNP diversity, it was observed overall (pairwise SNP differences per kb) variation 3.93, and on average 2.58 within *indica* subspecies, 1.96 within *japonica* and 5.9 between *indica* and *japonica* (Xu *et al.,* 2012; Duitama *et al.,* 2015). Supporting the earlier report that subspecies *indica* has more genetic diversity than japonica.

It is obvious to increase approximately 25% rice grain of the present production to meet up the demand of population growth in 2030 from less amount of arable land, less water and under adverse effects of climatic change (Flood/drought/salinity). Estimated annual yield increase is needed about 1.2–1.5%, which means yield increase of 0.6 t/ha world-wide on an average (Seck *et al.,* 2012). Population growth will reach nine billion (9 Billion) by 2050, and it is imperative to increase the food supply to meet up the demands (Godfray *et al.,* 2010).

Genomic Assisted Breeding for Rice Crop Improvement

Molecular genetics can play a vital role for the improvement of rice yield and productivity in the post-genomics era to sustain world food security (Miura *et al.* 2011; Huang *et al.* 2013). Grain yield enhancing processes are the main target to increase yield in the rice breeding and genomic research (Huang *et al.,* 2013; Zuo and Li, 2014; Li *et al.,* 2011). Advanced genomic tools are being used for study the mapping population and functional genomics (SNP based marker-trait association) analyses to dissect the unknown complex traits (*QTLs*) to uncover the *QTLs*/genes (*GS3, GW2, qSW5/GW5, GS5, TGW6* and *GIF1*) for grain size/weight in rice (Fan *et al.* 2009; Zhang *et al.* 2012; Weng *et al.,* 2008; Zhao *et al.,* 2011; Huang *et al.,* 2010). The GWAS have successfully identified many *QTLs*/gene for agronomic traits, including grain yield (Li *et al.,* 1997; Venuprasad *et al.,* 2012), flowering time (Chen *et al.,* 2014), plant height (Ashikari *et al.,* 2002), aluminum tolerance (Famosa *et al.,* 2011), grain yield under drought stress, and submergence tolerance (Xu and Mackill, 1996) in rice. Genotyping-by-sequencing (GBS) was used to discover and call SNPs on 369 advanced inbred breeding lines of rice for grain yield (kg/ha), flowering time (days to 50% flowering), and plant height (cm) (Spindel *et al.,* 2015).

Plant height specific *QTLs* were identified based on genomic breeding (sequencing based) in rice (Huang *et al.,* 2009), which was considered as 'Green revolutionary' gene. Whole genome sequencing of rice cultivar is performed to identify gene specific markers (may be SNPs) which may help in marker assisted selection (MAS) in breeding program. Amylose content determining gene specific SNPs were detected in rice varieties using whole genome re-sequencing method and discovered three SNPs (Waxy-1, Waxy-2 and Waxy-3) (Duitama *et al.,* 2015) for quality improvement and reported as amylose

content marker in rice by other group (Larkin and Park, 2003). Many gene/ *QTLs* were identified using cloning and functional analysis of the genes based on comparative genome characterization (Weng *et al.,* 2008; Jiang *et al.,* 2012). The *QTL* related to grain yield DEP1 was characterized and responsible for grain number per panicle and for erect panicle (Huang *et al.,* 2009). GS3 is major *QTL* related to grain weight and size was identified by Fan et al (2006). Heading date *QTL* Ghd7 and days to heading (DTH8) were identified in rice (Xue *et al.,* 2008; Wei *et al.,* 2010; Huang *et al.,* 2013). Wild species *O. rufipogon* has also been resequenced for the generation of functional SNPs, which can be used to improve the crop varieties and relationship between rice diversity and domestication of rice crop (He *et al.,* 2011; Huang *et al.,* 2012a). The 'Oryza Map Alignment Project' (OMAP) was developed for alignment of sequencing data of wild rice based on reference genome to identify genes and *QTLs* (Wing *et al.,* 2005).

RIL lines are re-sequenced using the NGS technique and ultra-high-density linkage map has been constructed for the identification of many *QTLs* associated with yield and gene (DTH8 and LAX1). The fine mapping of *QTLs* and genes can accelerate the *QTL* cloning and molecular breeding to develop improved rice varieties (Gao *et al.,* 2013). Resequencing based gene mapping has been applied to identify some important gene/*QTLs* (qPH1, qPH5, qGL-3, qGN1) from the rice lines (Wang *et al.,* 2011; Gao *et al.,* 2013; Yu *et al.,* 2011; Duan *et al.,* 2013). The International Rice Functional Genomics Project (IRFGP) has been initiated to determine the function of each of the alleles in the rice genome (Ikeda *et al.,* 2013). Bacterial blight (BB) is one of the most devastating diseases of rice which causes huge loss in rice production worldwide and caused by *Xanthomonus oryzae* pv. *oryzae* (*Xoo*). Different approaches have been applied to make BB resistant rice varieties through molecular breeding. Some BB resistance genes (*Xa3/Xa26, Xa4, Xa4b, Xa6,* and *Xa9*) have been transferred to improved varieties through MAS breeding. These genes are

located at the end of the long arm of chromosome 11. The receptor-like kinase gene was found in the cloned region of *Xa3/Xa26* gene (Sun *et al.,* 2004) based on GWAS.

The genetic improvement of Basmati rice for yield, quality and resistance to bacterial leaf blight (*Xa21, xa4, xa13, xa5, Xa33t, xa34t* and *Xa38*) and blast (*Pi1, Pi2, Pi5, Pi9, Pi54, Pib, Piz, Piz5, Pi-ta* and *Pi54/Pi-K^h*), brown plant hopper [*Bph-3/17/18/20/21* and *Bph18(t)*], sheath blight (*qSHB*) and gall midge (*Gm4* and *Gm8*) diseases has been performed by pyramiding the multiple genes/*QTLs* through marker-assisted back-crossing (MABC)/marker-assisted foreground and background selection (Cheema *et al.,* 2008; Madhavi *et al.,* 2011; Natarajkumar *et al.,* 2012; Sujatha *et al.,* 2013; Pandey *et al.,* 2013; Pradhan *et al.,* 2015). Introgression of known cloned genes and *QTLs* for drought tolerance (*DTY1.1, DTY2.1, DTY2.2, DTY3.1, DTY3.2, DTY9.1* and*DTY12.1*), flood tolerance (submergence) (*Sub1*) and salinity stress (*Saltol*) tolerance are initiated to develop high-yielding mega rice varieties (ADT46, Bahadur, MTU1075, Pooja, Rajendra, Mahsuri, Ranjit, ADT39, Pusa44, ADT45, Gayatri and Savitri) of India through MAS (http://india.irri.org/mega-projects-in-india, Singh *et al.* 2015). This eventually may lead to development of certain diverse genetically-tailored high-yielding and climate resilient early maturing Indian rice varieties for sustaining food security.

Furthermore, millions of single nucleotide polymorphism (SNP) and insertion-deletion (InDel) markers have already been identified in rice. Saturation of the genome with such sequence based SSR and SNP markers is accelerating fine mapping and map-based cloning of genes, and thus, development of gene-based allele-specific markers. Rice improvement programs are expected to benefit greatly from the use of these markers in near future. The availability of gold standard reference genome sequence of *japonica* rice cv. Nipponbare (International Rice Genome Sequencing Project 2005) has propelled the genome resequencing and transcriptome sequencing of diverse rice

genotypes in recent years by use of NGS (next-generation sequencing) approaches in India. This in turn led to the development of enormous resources in the form of genomic (genic) simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers at a genome-wide scale in rice. For instance, non-redundant 2495052 SNP and 324034 InDel markers have been discovered by comparing the NGS-based whole-genome resequencing data of six elite *indica* inbred lines (three of each cytoplasmic male sterile and restorer lines) to accelerate genomics-assisted breeding for hybrid performance in rice (Subbaiyan *et al.,* 2012). Subsequently, the whole genome resequencing of three drought/salinity tolerant (Nagina 22 and Pokkali) and sensitive (IR64) rice accessions identified non-redundant 1784583 SNPs and 154275 InDels between reference Nipponbare and three resequenced rice accessions. Based on this outcome, genome-wide 401683 SNPs between IR64 and Pokkali and 662509 SNPs between IR64 and Nagina 22 that are well-distributed across coding and non-coding regions of these sequenced genomes were discovered with the eventual aim to deploy them in marker-assisted breeding for abiotic stress tolerance in rice (Jain *et al.,* 2014). More recently, the comparison of whole genome resequencing data of a widely cultivated low glycemic index-containing *indica* rice variety, Swarna, with reference genome Nipponbare, identified 1,149,698 SNPs (65,984 non-synonymous SNPs) and 104,163 InDels for deciphering the genetic basis of complex glycemic index quantitative trait in rice (Rathinasabapathi *et al.,* 2015).

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Review Article

Nitric oxide and calcium signalling in plants under salinity stress and their crosstalk

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Abstract

Salinity is considered as one of the major factor affecting the crop production throughout the world. The oxidative stress induced by salinity can retard plant growth and yield as major part of energy is wasted on conserving water and improving ionic balance. The free radicals produced during stress are considered to be a major factor for most of the damages as these free radicals attack vital biomolecules such as lipids, protein and carbohydrates which are the basic requirement of almost all physiological and developmental processes. Understanding the mechanism of stress tolerance along with the involvement of important signalling molecules in stress signalling network is essential for crop improvement. Likewise, the two signalling molecules nitric oxide and calcium ion have been reported to be actively involved in upregulation of various stress response mechanisms thus indicating the existence of a possible cross talk among these molecules and other associated pathways. In this review, emphasis was given on the impact of salinity and oxidative stress mediated damages on plant system. Additionally, the role of nitric oxide and calcium ion as signalling molecules in response to stress signals and their implication in mitigation of salinity stress has also been discussed.

Keywords: Calcium ion, Free radicals, Nitric oxide, Salinity, Signalling.

Introduction

Salinity is considered as one of the major factor affecting the crop production throughout the world. Salinity either in water or soil represents one of the major abiotic stresses especially in arid and semi-arid regions, which can severely limit the agricultural production (Shanon, 1998). High concentration of salt creates ionic imbalance and hyper osmotic stress in plant system which consequently leads to oxidative damages. Such drastic changes in plant system cause retardation of growth, molecular damages, membrane disruption and even death. For the plant to be tolerant to salinity stress: their homeostasis must be re-established along with detoxification mechanism must be boosted (Zhu, 2001). Most of the cellular damages caused by salinity are usually associated with ROS mediated oxidative stress (Parida and Das, 2005).

Nitric oxide and calcium both are considered as highly versatile signalling molecules. Various literatures have reported

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the significant involvement of both of these molecules in wide range of physiological and developmental processes in plants. Additionally, these molecules have found to mitigate the adverse effect of varied environmental stresses including salinity (Wilson *et al.*, 2008; Sirova *et al.*, 2011; Lecourieux *et al.*, 2006).

Effect of salinity on plant system

The two major consequences of salinity on plant system are osmotic stress and ionic toxicity; these physical conditions affect all other physiological, biochemical and developmental processes in plants (Yadav *et al.*, 2011). High salt content in the substratum creates rise in osmotic pressure of the substratum thus, affecting the water uptake capacity of plants. Furthermore, decrease in the turgor pressure of the plant cells cause closing of stomata which leads to reduced carbon fixation but increase in ROS production. These highly reactive and unstable free radicals disrupt various cellular processes by damaging the major biomolecules like lipids, proteins, and nucleic acids (Parida and Das, 2005). Ionic toxicity is the physiological state

in which the equilibrium of ions is disturbed which causes perturbation in cellular metabolism and processes. High concentration of sodium ions at the surface of the root disrupts plant nutrition by inhibiting both K^+ uptake and enzymatic activities within the cell (Aslam et al., 2011). Potassium is an important nutrient which regulates huge number of enzymes activities associated with various major pathways (Kader and Lindberg, 2010); on the other hand, sodium ions inhibit the activity of enzymes. Na^+ is a cation almost similar to K^+, for this reason Na^+ can cross the cell membrane without much disturbance (Parida and Das, 2005). As suggested by Rodriguez-Navarro, (2000) optimum concentration of K^+ required is 100-200mM in the cytosol and the concentration of cytosolic Na^+ excess of 10mM creates stress environment in the system. The oxidative stress induced by salinity can retard plant growth as major part of energy is wasted on conserving water and improving ionic balance (Kader and Lindberg, 2010).

Strategy for prevention of Na^+ toxicity in plants

In order to overcome salt stress, plants have developed different strategies for their survival. For instance, for combating Na^+ toxicity most of the glycophytes depend on restriction of Na^+ intake, but this strategy is successful to some extent only because of the electronegative environment in inner cellular system. Additionally, the cation transporters are fairly permeable to Na^+, therefore the constant influx of Na^+ along the electrochemical gradient is not terminated completely (Amtmann et al., 1999). But interestingly, halophytes overcome this ion toxicity by coupling the uptake of ions via roots with the compartmentation of ions into cellular vacuoles (Hasegawa et al., 2000; Blumwald et al., 2000).

Concept of free radicals

A free radical is defined as a molecular species which is capable of independent existence and posses an unpaired electron in its outermost atomic orbital. This unpaired electron results in presence of certain common properties that are shared by most of the radicals. These free radicals are highly unstable as well as highly reactive. They have the capability to either donate an electron or accept an electron from other molecules, therefore altering their native properties (Cheeseman and Slater, 1993; Lobo et al., 2010). The free radicals generated from oxygen are called reactive oxygen species (ROS) and those from nitrogen are termed as reactive nitrogen species (RNS). ROS includes various forms of activated oxygen molecules, such as superoxide ($O^{2 \cdot -}$), hydroxyl ($^{\cdot}OH$) and peroxyl ($ROO^{\cdot}$), as well as non-free radicals hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Likewise, RNS includes nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$) and non-free radicals such as nitrous acid (HNO_2) as peroxynitrite ($ONOO^-$) (Halliwell, 1994; Chanda and Dave, 2009). These free radicals are generated under normal physiological conditions but become harmful when not being eliminated from the cellular systems. In fact, such imbalance between the production and elimination of reactive oxygen species in the cell system leads to a condition known as oxidative stress. After excessive accumulation, they attack vital biomolecules leading to cell damage and homeostatic disruption. The major targets of these free radicals are lipids, nucleic acids, proteins and carbohydrates (Aruoma, 1994). The formation of free radical is a consequence of both enzymatic and non-enzymatic reactions which occurs continuously in the cell system. Enzymatic reactions include those phenomena involved in the phagocytosis, respiratory chain, synthesis of prostaglandin also in the cytochrome P450 system (Lui et al., 1999; Lobo et al., 2010). Free radicals can also be produced in non-enzymatic reactions between oxygen and organic compounds as well as those initiated by ionizing reactions.

Importance of Nitric oxide signalling in plant system

Nitric oxide (NO) is an important signalling molecule, which has been known to participate in wide spectrum of regulatory functions in almost all stages of plant development (Wilson et al., 2008; Sirova et al., 2011). In the year 1975 the emission of NO from plants was first observed by Klepper in 1975, in soybean plants treated with herbicides (Klepper, 1979).

Plants not only react to the atmospheric or soil NO, but they are also able to generate NO via reduction of apoplastic nitrite (Bethke *et al.*, 2004) or by carotenoids in presence of light (Cooney *et al.*, 1994). The major production of NO in plants, however, is probably carried through the action of NAD(P)H-dependent nitrate reductase enzyme (Dean and Harper, 1988) which is also considered as an endogenous source of NO in plant system (Yamasaki *et al.*, 1999).

The synthesis of NO in animals is carried out by the enzyme nitric oxide synthase (NOS) via deamination of L-Arginine. But, there are no such genes in plant system including *Arabidopsis thaliana* that allow homology with *NOS* genes of animals (Gupta *et al.*, 2011). Among the photosynthetic members, only *Ostreococcus tauri*, an unicellular green alga was found to possess a NOS having a homology of only 45% with the human NOS (Foresi *et al.*, 2010). At present several pathways involved in NO synthesis in plant system are known, also some are assumed which are given in fig. 1. The biosynthetic pathway leading to the production of NO in plants might be either oxidative or reductive. The oxidative pathway is carried out by NOS like enzyme which also includes synthesis from polyamines. The reductive pathway is mediated by enzymes such as nitrate reductase (NR) and nitrite–NO reductase (Ni-NOR). Furthermore, this pathway includes xanthine oxidoreductase (XOR) in peroxisomes and cytochrome *c* oxidase (COX) that synthesizes NO from nitrite in mitochondria (Mamaeva *et al.*, 2015).

The application of exogenous NO to plants or cell cultures has revealed valuable information about the influence of this molecule on various physiological and biochemical processes. The summary of the functions NO associated with various physiological, biochemical and molecular processes is given in fig. 2. The earlier reports suggest that NO can mediate the biological effects of signalling molecules such as phytohormones. The biosynthesis of NO has been found to be induced by cytokinin in different plants and hence the possibility of involvement of NO in the cytokinin-induced programmed cell death process is proposed by Neill *et al.*, (2003). Likewise, it has been demonstrated that NO synthesis in cucumber roots is induced by auxin (Pagnussat *et al.*, 2003). Additionally, the interaction between both the gaseous molecules NO and ethylene in the maturation and senescence of plant tissues has been reported during plant development (Lamattina *et al.*, 2003).

The identification of the NO synthesis enzymes and the discovery of regulatory role of NO in the activity of specific proteins within sub-cellular compartments provided significant understanding of NO signalling at the molecular level (Hanafy *et al.*, 2001; Kone *et al.*, 2003; Stuehr *et al.*, 2004). Over the past decade, considerable progress has been made in understanding the mechanism of NO signalling in plants. NO modulates the activity of most proteins through nitrosylation and tyrosine nitration mechanism. The post translational modifications via nitrosylation as well as S-nitrosylation have been resulted in regulation of several plant proteins *in vitro* and also *in vivo* to some extent. The proteins which are the targets of NO include haemoglobin, cytochrome c oxidase, metacaspase 9, glyceraldehyde-3- phosphate dehydrogenase, and methionine adenosyltransferase (Besson-Bard *et al.*, 2008). Endogenous NO has been found to function as a calcium ion-mobilizing messenger by inducing the rise in cytosolic Ca^{2+} concentrations. The rise of cytosolic Ca^{2+} concentration further aid NO to modulate the protein kinases and channels involved in the signalling cascade, thus regulates important physiological processes such as stomatal closure, adventitious root formation an also the expression of defense genes (Garcia-Mata *et al.*, 2003; Lamotte *et al.*, 2006). In *Arabidopsis*, it was demonstrated that the production of NO by elicitors such as lipopolysaccharides is regulated by Ca^{2+} influx mediated by the cyclic nucleotide-gated channel (Ali *et al.*, 2007). The interplay of both NO and Ca^{2+} and their involvement in the plant acclimation during salinity stress also preventing the oxidative stress mediated damages and their adverse effects have been elucidated in fig. 4.
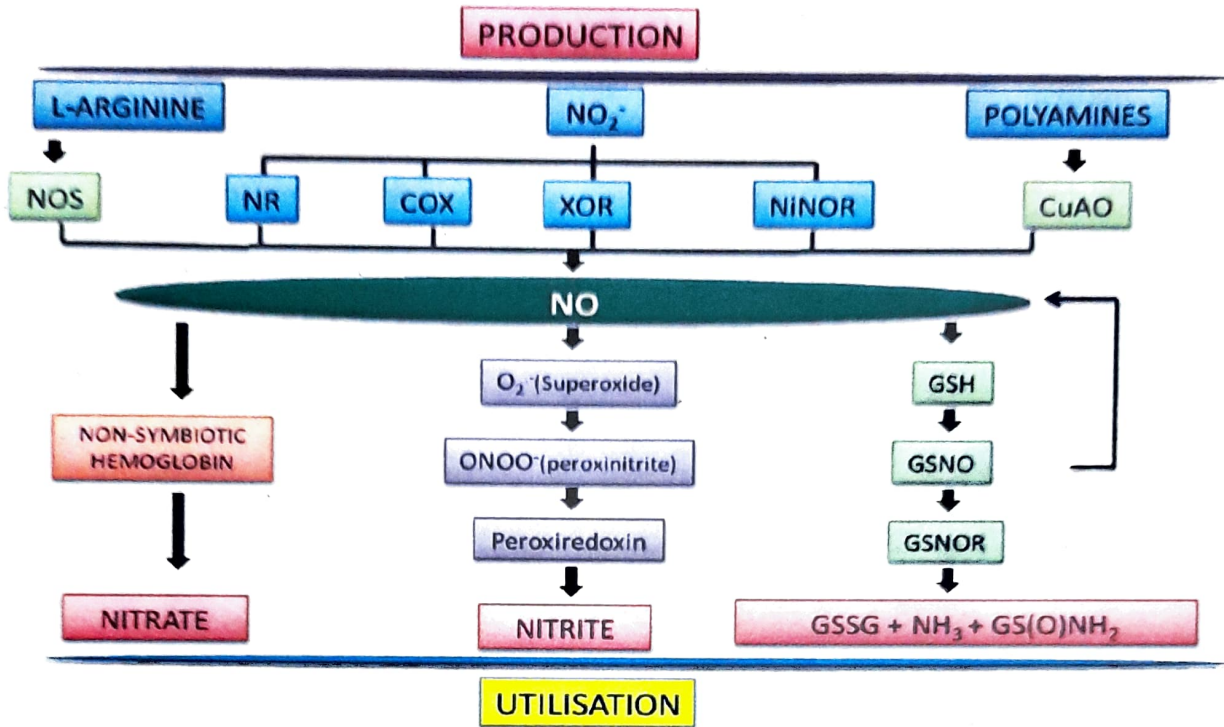
Fig. 1: Pathways involved in synthesis and utilization of NO in plant system (Mamaeva *et al.,* 2015).NOS: Nitric oxide synthase; NR: Nitric reductase; COX: Cytochrome oxidase; XOR: xanthine oxidoreductase; CuAO: Cu-amine oxidase; NiNOR: Nitrite-NO reductase; GSH: Reduced glutathione; GSNO: S-nitrosoglutathione; GSNOR: S-nitrosoglutathionereductase; GSSG: oxidized glutathione; GS(O)NH₂: glutathione sulfonamide.
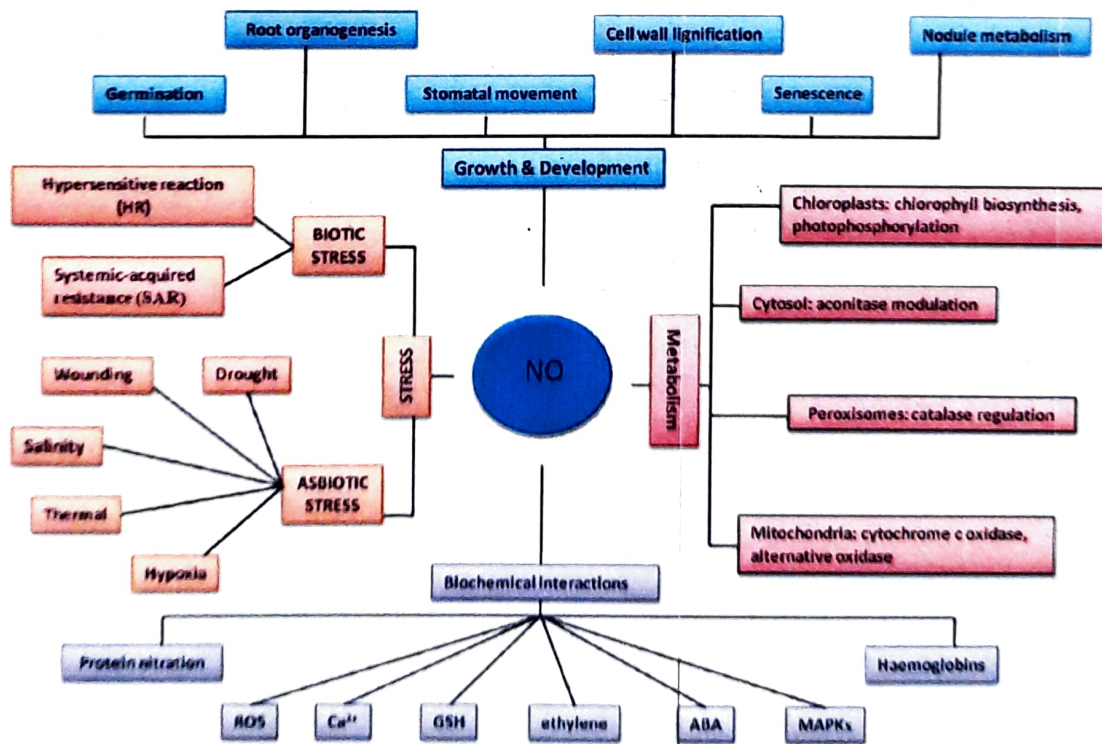

Fig. 2. Functions of Nitric oxide associated with various physiological, biochemical and molecular processes (del - Rio et al., 2004).

Role of Calcium signalling in plant system

The calcium ion has been well established as a second messenger in several plant signalling pathways, conveying a wide range of stimuli to appropriate physiological responses. Ca^{2+} signals are considered as a core regulator of cell physiology and cellular responses of plants to the environment. Many extracellular and environmental signals including both abiotic and biotic factors, elicit change in the cellular level of calcium, termed as calcium signatures (Lecourieux *et al.*, 2006). This "Ca^{2+} signatures" represent a central mechanistic principle for stimulus-specific information in the cellular system. The channels, pumps, and carrier proteins serve as the mechanistic basis for generation of Ca^{2+} signals by modulating the flux of calcium ions among the sub-cellular compartments, cell and its extracellular environment (Dodd *et al.*, 2010).The disorders due to Ca-deficiency in plants have been considered to be very much harmful in horticulture sector commercially (Shear, 1975). Some of the diseases caused due to deficiency of calcium in plants are tip burn and brown heart in leafy vegetables, blossom end rot of tomato fruit, empty pod in peanut also structural weakness in cell wall. The Ca-deficiency generally occurs when there is unavailability of sufficient calcium in the developing tissues due to failure of calcium mobilization by phloem. On the other hand, presence of excess calcium in the substratum also creates a cytotoxic environment for plants. The excessive calcium reduces the germination rate of the seeds and also retards the plant growth rates (Shear, 1975; White and Broadley 2003). The other functions of calcium ion in the plant systems are elucidated in fig. 3.

Since the presence of higher calcium ion concentration is cytotoxic, a sub micromolar level of calcium ion is maintained by Ca^{2+}ATPases and H^+/Ca^{2+} antiporters in unstimulated cells (Sze *et al.*, 2000; Hirschi, 2001). These proteins maintain this optimum level by fluxing the extra cytosolic Ca^{2+} either to the apoplast or the lumen of vacuole or endoplasmic reticulum (Sanders *et al.*, 2002). There are other class of proteins which change their conformation or catalytic activity upon binding with the calcium ion and hence regulate the calcium signals. Also it has been reported that specific sensors and signals of calcium ion signatures regulated cellular responses to specific biotic and abiotic stimuli (White, 2000).

The proteins responsible for the perception and decoding of Ca^{2+} signals are present in the cytosol and nucleus of the plant cell. Several calcium sensors with different Ca^{2+} binding characteristics, subcellular localizations and signalling interactions comprises a toolkit that helps in decoding the information within Ca^{2+} signatures in the form of spikes or oscillations (Dodd *et al.*, 2010; Batistic and Kudla, 2012). Further these sensor proteins accordingly carry the processing of this information into respective alterations in cell function. Conceptually, plant Ca^{2+} sensor proteins that are functionally signalling components have been classified into sensor relays and sensor responders (Sanders *et al.*, 2002). The sensor responder proteins which include Ca^{2+}- dependent protein kinases (CDPK) combine both sensing function and responding function, regulated by calcium-binding proteins that often cause conformational changes (e.g., protein kinase activity) within a single protein. Consequently, these kinases mediate the information encoded in Ca^{2+} signals into phosphorylation events of specific target proteins. In contrast, sensor relay proteins such as calmodulin (CaM) and calmodulin like protein (CML) also contain multiple calcium-binding domains and undergo conformational changes with Ca^{2+} signals but lack the enzymatic function. Therefore, these proteins have to interact with other target proteins and regulate their activity for transduction of Ca^{2+} signal, which means they must undergo Ca^{2+}-dependent protein-protein interactions (Luan *et al.*, 2002). The calcineurin B-like (CBL) protein are another family of sensor proteins which lack the enzymatic activity hence belong to sensor relay proteins. However, their specific interaction is with a family of protein kinases designated as CBL-interacting protein kinases (CIPKs), so, CBL–CIPK complexes are considered as bimolecular sensor responders (Hashimoto and Kudla, 2011). CaM is highly conserved in all eukaryotic members, whereas
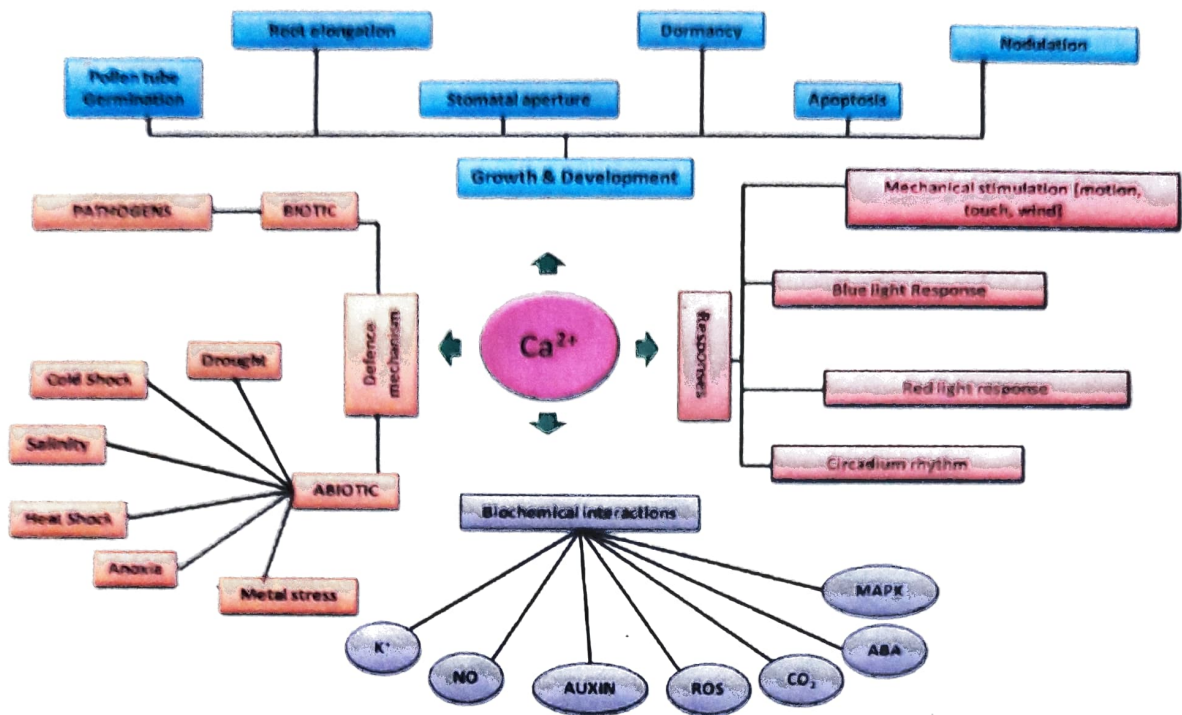
Fig. 3. Involvement of $Ca^{2}+$ signal in various physiological, biochemical and molecular processes in plant system (modified from White and Broadley, 2003; Leucouriex *et al.*, 2006)
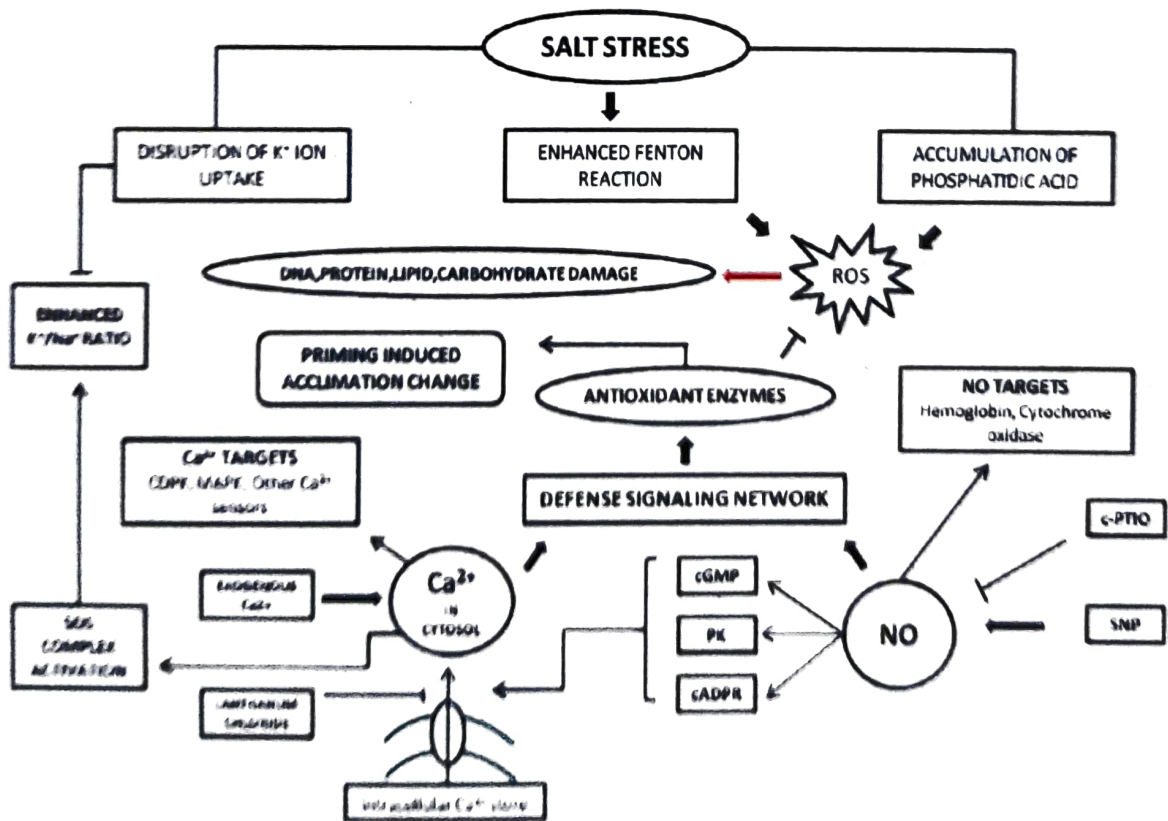

Fig. 4. Interplay of Nitric oxide and Calcium ion and their role in alleviation of oxidative stress mediated damages under salinity stress.

CML, CDPK and CBL proteins have been found to be present only in plant system (Batistic and Kudla, 2009).The specific binding of Ca^{2+} with Calmodulin7 (Cam7) results in direct interaction and regulation, while other calmodulins are likely to mediate gene regulation via interacting with other transcriptional (co)regulators. Metabolic and biosynthetic processes such as brassinosteroid synthesis are important targets of direct Ca^{2+}-dependent modulation (Du and Poovaiah, 2005), but on the other hand Ca^{2+}-dependent phosphorylation and gene regulation provides the major cellular currencies for transduction of specific Ca^{2+} signals into targeted downstream responses (Harper and Harmon, 2005).

Implication of NO for maintenance of redox homeostasis during salinity stress

NO is said to possess considerable capacity to regulate oxidative stress mediated damages along with the level and toxicity of ROS.

The properties of NO which makes it capable to exert a protective function against oxidative stress mediated damages as suggested by Yadav (2010) are given below:

i. It reacts with lipid radicals and stops the propagation of lipid oxidation.

ii. Scavenging the superoxide anion and formation of peroxynitrite which is toxic for plant are later neutralized by ascorbate and glutathione.

iii. Involvement in the activation of antioxidant enzymes.

The lupin seeds when subjected to sodium nitroprusside (SNP) treatment showed better germination under saline stress as well as heavy metal stress (lead and cadmium) suggesting involvement of NO in auxin signalling pathway (Kopyra and Gowdz, 2003). Later, Zheng *et al.* (2009) demonstrated that pre-soaking of wheat with SNP for 20h prior to germination resulted in increased germination rate and radicle weight under 300 mM NaCl. Additionally, decrease in Na^+ concentration but increase in K^+ concentration in the seeds were observed thereby indicating role of NO in maintaining a balance between K^+ and Na^+ during germination under salt stress. The pre-treatment of citrus root with exogenous SNP for a duration of 48h exhibited induction of primary antioxidant responses in the leaves of citrus subjected to salinity stress. The study revealed that SNP pre-treatment enhanced the activity of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) also prevented the NaCl-dependent protein oxidation (Tanou *et al.*, 2009). Zheng *et al.* (2009) claimed in their study that NO treatment effectively contributed to better accumulation of ferritin, a protein active in chelation of excess of ferrous ion present in the cellular system of barley plant subjected to salt stress. Exogenous application of NO through different modes have reported to be effective in regulating the functioning of photosynthetic pigments (Ruan *et al.*, 2002;), improving salt tolerance by modulating proton pump activity in maize (Zhang *et al.*, 2006), regulating osmotic balance and proline metabolism in tobacco (Ke *et al.*, 2013) and prevention of mitochondrial oxidative damage (Zheng *et al.*, 2009). Furthermore, various evidences have been provided by researchers about the protective effect of NO during other stress conditions besides salinity; alleviating the negative effects of UV radiations in wheat seedlings (Yang *et al.*, 2013); mitigating the oxidative injuries under heavy metal stress in lupin seeds (Kopyra and Gowdz, 2003) and modulating the metabolism of biochemicals during osmotic stress (Ke *et al.*, 2013). Other beneficial effects of NO donor reported are regulation of seed germination in *Senna macranthera* (da Silva *et al.*, 2015); maintenance of optimum Na^+/K^+ ratio in cotton seedlings (Dong *et al.*, 2014); enhancement in the enzymes involved in nitrogen metabolism namely nitrate reductase and nitrite reductase in tomato (Manai *et al.*, 2014) also reduction of lipid peroxidation, hydrogen peroxide and superoxide anions; elevation in the activity of major antioxidant enzymes accompanied with increase in the accumulation of biochemicals such as proline, glutathione and sugars under salnity stress in numerous plant system (Hayat *et al.*, 2012; Dong *et al.*, 2014; da Silva *et al.*, 2015;Hameed *et al.*, 2015; Ahmad *et al.*, 2016).

Multifunctional response of calcium in plant system during salinity stress

Calcium is considered as multifunctional element in plants besides as a nutrient, it is involved in several physiological processes like maintenance of membrane integrity, cell wall structure, increasing the activity of key enzymes and phytohormones interaction (Barker and Pilbeam, 2007). Additionally, it plays vital role in signalling network as a secondary messenger under varied environmental conditions (Tuteja, 2009; Batistic and Kudla, 2012). By virtue of this property it is capable of ameliorating the adverse effects of abiotic stresses including chilling, thermal, drought, heavy metals and salinity (Ma *et al.*, 2005; Shao *et al.*, 2008; Siddiqui *et al.*, 2011; Zehra *et al.*, 2012).

Many authors have suggested the beneficial role of calcium ion in the alleviation of the adverse effects of abiotic stress conditions. Therefore, the maintenance of optimum supply of calcium in saline soil is considered as an important factor in preventing the severity of specific ion toxicities, in those crops which are susceptible during salinity stress injury (Grattan and Grieve, 1999). In their study Hasegawa *et al.*, (2000) suggested that during salt stress, plants are able to tolerate high saline concentration by inducing the signal transduction cascades involving calcium ion. Thus, when exposed to stress conditions including salinity plants increase the cytosolic Ca^{2+} accumulation to combat the oxidative damages. Although the basic mechanism involved has remained unexplained, prevailing models for Ca^{2+} functioning include both membrane stabilisation and signalling significance. Considering the potential role of calcium ion in overcoming the negative impacts of several stresses, it has been implemented in various modes in order to provide stress tolerance to plants. Jaleel *et al.*, (2007) demonstrated that when *Catharanthus roseus* plants were supplemented with calcium chloride under drought condition, calcium ion provided osmoprotection to the plants along with increase in glycine betaine accumulation and indole alkaloid content in the shoot and roots of the plant. Also, a significant enhancement in the activity of antioxidant enzymes namely superoxide dismutase, catalase and peroxidase was reported in the same plant subjected to salinity stress (Jaleel *et al.*, 2007). According to Khan *et al.*, (2009) when calcium chloride was applied to linseed in combination with gibberellic acid proved more effective in ameliorating the negative effects of NaCl stress. It was found that the electrolyte leakage of membranes was reduced considerably with decrease in the accumulation of lipid peroxides and hydrogen peroxide. Later Sharma and Dhanda, (2015) suggested the protective role of calcium chloride treatment in *Vigna radiata* in which it was found that the presence of calcium ion helped in maintenance of photosynthetic pigments under salt stress. Similarly, calcium was found to maintain the rate of photosynthesis in *Zoysia japonica* under drought stress by reducing the damage of photosynthetic pigment (Xu *et al.*, 2013); increasing the germination rate and growth of forest strand under simulated acid rain (Liu *et al.*, 2011); involved in the enhancement of chilling stress in *Stylosanthes guianensis* by interacting with abscisic acid (Zhou and Ghou, 2009); the application of calcium in the culture medium was found to activate the accumulation of flavonol in *Polygonum hydropiper* (Nakao *et al.*, 1999); also increase in the activity of antioxidant enzymes, regulation of biochemical metabolism and maintenance of membrane integrity by calcium has been reported in plant system under various stress conditions (Jaleel *et al.*, 2007; Khan *et al.*, 2009; Zhou and Ghou, 2009; Xu *et al.*, 2013; Sharma and Dhanda, 2015).

Crosstalk between Nitric Oxide and Ca^{2+}

The complex cross-talk between NO and Ca^{2+} involve components of Ca^{2+} signalling machinery modulated by NO-dependent mechanisms at post-translational and/or transcriptional levels (Besson-Bard *et al.*, 2008). It is considered that NO regulates the overall control of Ca^{2+} homeostasis by regulating almost all types of associated Ca^{2+} channels and transporters. NO controls the Ca^{2+} homeostasis either via S-nitrosylation of the concerned proteins or through other second messengers namely, cGMP and cyclic ADP ribose (cADPR) (Willmott *et al.*, 1996;

Clementi, 1998 ; Stamler *et al.*, 2001; Ahern *et al.*, 2002; Hess *et al.*, 2005) (Fig. 4).

cADPR is a Ca^{2+} mobilizing messenger that induces release of Ca^{2+} from intracellular Ca^{2+} stores into various plant cells by activating the Ca^{2+} permeable channel ryanodine receptors (Allen *et al.*, 1995; Fliegert *et al.*, 2007). The rise in the cytosolic Ca^{2+} concentrations accompanied with an influx of Ca^{2+} from the extracellular space was observed in *Vicia faba* and *Nicotiana plumbaginifolia* when exposed to NO donors (Garcia-Mata *et al.*, 2003; Lamotte *et al.*, 2004). Previous studies suggested ryanodine receptors -like channels act as a main target of NO action and cADPR as key intracellular messenger responsible for mediating NO signals (Willmott *et al.*, 1996; Clementi, 1998). Interestingly, parallel investigation revealed that exogenous NO was unable to trigger any changes in Ca^{2+} concentration in *Nicotiana plumbaginifolia* cells (Lecourieux *et al.*, 2005). Therefore, these findings indicate that the regulation of Ca^{2+} homeostasis by NO might be restricted to specific cellular compartments.

Furthermore, studies on *Vicia faba* guard cells and *Nicotiana plumbaginifolia* cells revealed that protein kinase inhibitors significantly suppressed the NO mediated elevation of $[Ca^{2+}]cyt$ which indicates that besides cADPR involvement of protein kinases is also essential for signalling cascades that relay NO signals to Ca^{2+} channels (Sokolovski *et al.*, 2005; Lamotte *et al.*, 2006).

Interestingly, it has also been suggested that elicitor-induced NO production is enhanced by an upstream influx of extracellular Ca^{2+} (Lamotte *et al.*, 2006; Vandelle *et al.*, 2006). In agreement to above cited literature, a plasma membrane *Arabidopsis* cyclic nucleotide-gated channel (CNGC) member: CNGC2 was identified as a key Ca^{2+} channel which links the rise in Ca^{2+} influx to downstream NOS-like mediated NO production (Ali *et al.*, 2007). Further exploring the complexity of interplay between Ca^{2+} and NO, a study by Vandelle *et al.* 2006 suggested that NO might down regulate its own Ca^{2+}-dependent synthesis by inhibiting elicitor-induced influx of extracellular Ca^{2+}. This negative feedback mechanism is proposed as a strategy to protect the cells from the adverse effects of excessive accumulation of NO as well as Ca^{2+}.

The evidence summarized above documents the complexity of the interaction between NO and Ca^{2+}, but still a substantial effort is required to understand the mechanisms by which NO modulates fluxes of Ca^{2+} and its cellular homeostasis. Another unresolved issue concerns the impact of interplay between these two signalling molecules on the cell response.

Conclusion

Salinity management strategy is very essential for the production with better yield and quality of agricultural crops. Though some group of plants have developed defence mechanism against the salinity stress, but most of the agricultural crops are found to be susceptive. Therefore, several techniques have been suggested by the scientists for overcoming this problem and the implementation of signalling molecules and other metabolites as potent elicitors has showed considerable success in this field. The signalling molecules NO and Ca^{2+} due to their versatile characteristics are reported to be involved in almost all the processes in plant system. Among which their role in providing salinity tolerance to plants could be of great benefit to agricultural sector. From this review it can be suggested that NO and Ca^{2+} are not only stress signalling molecules but they have active role as intrinsic signal in developmental aspects. Furthermore, extensive study on genetics, proteomics and metabolomics along with additional physiological approaches are essential for better understanding of the mechanistic involvement of both NO and Ca^{2+} in the transduction pathways and their interplay among themselves, other factors and their perception and signal transmission to specific downstream responses.

Acknowledgement

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Research Article

In vivo seed germination and seedling morphology of Phoenix dactylifera L. and Phoenix sylvestris (L.) Roxb.

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Abstract

A comparative study of Phoenix dactylifera L. and Phoenix sylvestris (L.) Roxb. has been done in the Medicinal Plant Garden in North Bengal University. The germination status of mature seeds of two species in vivo condition was recorded. It was seen that Phoenix dactylifera prefers natural pH (45.5) where as Phoenix sylvestris prefer acidic soil with pH (6-7). During this study total seed output, times of germination, first aerial leaf, veneration pattern and reproductive capacity were calculated.

Key words: Seed germination, Seedling morphology, Phoenix dactylifera, Phoenix sylvestris, West Bengal, India.

Introduction

The family Arecaceae (Palmae) is one of the largest monocotyledonous family, comprising over 212 genera with about 2,779 species distributed worldwide (Basu and Chakraverty, 1994). Phoenix dactylifera L. and Phoenix sylvestris (L.) Roxb. belongs to the Subfamily Coryphoideae, tribe Phoeniceae of the family Palmae (Arecaceae). Basu and Chakraverty (1994) reported a total of 17 species of Phoenix are growing in different parts of the World. Species of Phoenix are growing in diverse habitats that occupy swamps, deserts, mangroves and coastal areas. Most of Phoenix species originate in semiarid region, but usually occur near high ground water levels, riverside or springs. About 92 species and 4 varieties of wild and semi wild palms representing of 21 genera are distribution in India. They are chiefly occurring in three major geographical regions, viz. Peninsular India, Eastern and North-Eastern India and Andaman & Nicobar Islands. A very few species of palm taxa are also occur in the rest parts of India, particularly in the sub-

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Himalayan valleys and plains of northern India, semi-arid parts of Western India, Gangetic plains, estuarine or mangrove forests of Ganga and Mahanadi delta, moist hilly tracts of Orissa, South and North Bihar (Basu and Chakraverty, 1994). Phoenix dactylifera and Phoenix sylvestris are distributed from Canary Islands through sub-tropical and tropical Africa, the Arabian Peninsula, the Indian subcontinent and Indo-china to Hongkong (Uhl and Dransfield, 1987). In India these palms are grown as ornamentals because of their beautiful showy structures. Apart from the ornamental use, both the species having great economic values due to their delicious nuts, sweet sap, timber etc.

Materials and Methods

Mature fruits of both the species have been collected from the natural sources during the month of July, 2015 and 20 numbers of seeds of each species were sown in pot with three replica. The standard pH for germination and growth of seedlings of each species were tested in laboratory. Progress of seeds germination were studied after regular interval. To record the seed behavior and seedling morphology, conventional methods were

Table 1: Seed shape, size and weight measurements

Taxa	Ave. Length (cm)	Ave. Breadth (cm)	Shape index	Size index	Seeds Weight (gm)	
					1 seed	100 seed
Phoenix dactylefera	1.86	0.94	1.98	1.75	1.34	134
Phoenix sylvestris	2.28	1.32	1.73	3.01	1.73	173

Table 2: Seed output and Reproductive capacity

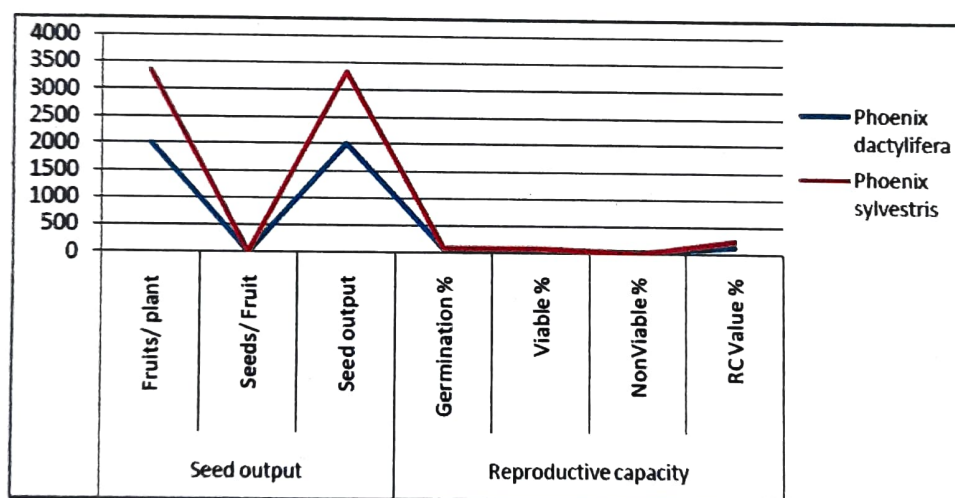| Taxa	Seed output			Reproductive capacity			
	Fruits/ plant	Seeds/ Fruit	Seed output	Germination %	Viable %	NonViable %	RC Value %
Phoenix dactylifera	2013	1	2013	71.67	71.67	28.33	131.15
Phoenix sylvestris	3341	1	3341	93.33	93.33	6.67	239.87

Fig. 1. Graph showing comparative study of Reproductive capacity and Seed output of *Phoenix sylvestris* and *Phoenix dactylifera*.

followed as suggested by Chowdhury (2009), Bose and Paria (2015).

This part of work also attempt to determined the average seed output and average seed germination for both the species. The average seed output of a plant is determined by taking 10 plants that were selected at random and counted separately.

Mean value is calculated for average seed output. The collected seeds were then dried out in air and stored in a desiccator. During seed count, number of fruit per plant, seeds per fruit also counted. Seed shape, seed colour and other seed morphology along with seed weight were also been noted. Seed shape and size, germination Percentage, reproductive capacity and seed output were calculated following the methods as suggested by Hill *et al.* (1986), Salisbury (1942) and Chowdhury (2009).

Result and discussion

Present study mainly focused on the entire seed morphology and reproductive capacity of *Phoenix dactylifera* and *Phoenix sylvestris* at garden condition at University of North Bengal (Figure 2). It was clearly recorded that the

seeds of *Phoenix dactylifera* preferred acedic (pH 6-7) soil for germination where *Phoenix sylvestris* prefer germination in sandy soil with basic (pH 45.5) condition. During this study it was found that the rate of seed germination of *Phoenix dactylifera* is very less where as *Phoenix sylvestris* showing good and satisfactory result. The first seedling leaf tip is more or less obtuse in *Phoenix sylvestris* whereas acute leaf tips in *P. dactylifera*. During germination in *P. dactylifera* the length of the remote tubule is on average 7cm whereas around 19 cm in *P. sylvestris* were recorded. It was also seen that first aerial leaf of the seedling of *Phoenix dactylifera* consists of 7 parallels veins where as *phoenix sylvestris* consists of 5 parallel veins with vigorous rooting system.

Fig. 2. Mature tree (A); seeds (C); seedlings (F); undivided main root system (J) of *P. sylvestris*; Mature tree (B); germinated seeds (D & E); seedlings (G & H); divided main root from the adjacent point (I) of *P. dactylifera*.

The details of the seed output, '%' of germination and Reproductive capacity for both the species were also calculated and given in Table 1 & 2. The entire calculated data shows that the *P. sylvestris* showing the higher seed production and also reproductive capacity (239.87%) than that of *P. dectylifera*

(131.15%) and the comparative analysis was given in fig. 1.

Conclusion

Further detail studies regarding the seedling morphology of *Phoenix dactylifera, P. sylvestris, P. paludosa, P. acualis* will be taken for better understanding of the taxa through seedling morphology. Because of the genus *Phoenix* needs a long time for flowering which is very much essential for taxonomical identification, moreover the genus *Phoenix* is also dioecious in nature, so the study of seed and seedlings morphology can help us to during the segregation of male and female plants at juvenile stage for cultivation for better yield.

Acknowledgment

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Research Article
In vitro production of diarrhoeal enterotoxin by Bacillus cereus isolates from milk and dairy products

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Abstract

Bacillus cereus is a great safety concern for dairy industry as it is associated with incidences of food poisoning by producing enterotoxins. In the present study, growth temperature profile and enterotoxin production potential of 144 strains of Bacillus cereus isolated from milk and dairy products were investigated. Out of them, 107 (74.3%) were able to grow at ≤7 °C. Presence of such a large number of psychrotolerant/psychrotrophic strains in dairy environment is of major concern mainly because of their potential for growth, spoilage and toxin production in chilled products. Out of 144 isolates, 134 (93%) exhibited β-haemolysis. While 98% of the isolates from milk and 89% from cheese were positive for diarrhoeal enterotoxin, all the isolates from milk powder, ice cream, paneer and butter were positive. The prevalence of potent producers of enterotoxin among dairy isolates poses a high health risk.

Key words: Bacillus cereus, Growth temperature requirement, Enterotoxin, Haemolysin, Dairy product.

Introduction

Endospore-forming bacteria are important contaminants in dairy industry and significantly affect the quality of milk and dairy products. Particularly, members of *Bacillus cereus sensu lato* (*s.l.*) are significant, as they are associated with foodborne outbreaks by producing enterotoxins (Anderson Borge *et al.*, 2001) as well as responsible for decrease in the organoleptic quality of milk and dairy products by causing spoilage, like sweet curdling and bitterness of milk (Chen *et al.*, 2003).

Bacillus cereus may enter into the dairy environment from various sources during production, handling and processing, mainly from improperly cleaned and sanitized equipments. The hydrophobic properties of endospores and their resistance towards heat, desiccation and disinfectants allow them to attach to processing equipment and survive cleaning procedures (Ryu and Beuchat, 2005). Adherence to stainless steel surfaces of dairy plants can result in biofilm formation which can be an important reservoir for recurrent contamination of milk and dairy products (Kumari and Sarkar, 2014a, 2016; Shaheen *et*

al., 2010). With an increasing demand of milk and dairy products, the need of extended refrigerated storage of raw milk before processing and the application of higher pasteurization temperatures for prolonged shelf-life requirements leads to serious concerns, like tolerance, adaptation or resistance of spores or vegetative cells to conditions of low temperature or low pH that were previously presumed to stop growth, or to treatments such as ultrahigh heat treatment (UHT) that were expected to inactivate all living materials (Heyndrickx, 2011). Thus, it has become important to characterize spore-formers in the dairy sector.

The incidence of foodborne illnesses has increased globally, and it becomes more important in developing countries where food products are frequently exposed to contaminated environments in food processing industries and temperature abuse during transportation and storage at retail outlets (WHO, 2007). Hence, *B. cereus* is an important safety and shelf-life concern in dairy industry. It is associated with two types of food poisoning: the diarrhoeal type by enterotoxin production in the small intestine and the emetic type by toxin which is formed in food. The rapid onset of the emetic disease, generally within 5 h after consumption of a

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meal, indicates that this is due to a toxin preformed in the food. This toxin is not inactivated during food processing or gastrointestinal passage due to its high resistance against heat treatments, pH extremes and proteolytic degradation (Rajkovic et al., 2008). Diarrhoeal syndrome, which develops 8-16 h after ingestion of the contaminated food, has been linked to two enterotoxin complexes, haemolysin BL (Hbl) and nonhaemolytic enterotoxin (Nhe), and a single protein, cytotoxin K (CytK) (Lund et al., 2000; Stenfors Arnesen et al., 2008). Although B. cereus is mainly associated with gastrointestinal disorders, it is an opportunistic human pathogen associated with a multitude of other infections, such as severe eye infections, periodontitis, necrotizing fasciitis, endocarditis, nosocomial acquired bacteraemia, osteomyelitis, sepsis, liver abscess, pneumonia and meningitis, particularly in postsurgical patients, immunosuppressed individuals, intravenous drug abusers and neonates (Ramarao and Sanchis, 2013).

Since pathogenic potential of the different B. cereus isolates is highly variable, from nontoxic to highly toxic strains, and toxin expression is influenced by food components, temperature and other environmental factors, there is need to study toxigenic potential of strains within the relevant food products. Therefore, aim of this research was to study the growth temperature limits and enterotoxin production potential of strains, previously isolated from milk and dairy products.

Materials and methods

Microorganisms

A total of 144 strains of B. cereus, isolated from milk and dairy products (Kumari and Sarkar, 2014b), were evaluated for growth temperature limits and production of enterotoxins.

Growth temperature requirement

Growth temperature was determined by inoculating J-broth, supplemented with 1 g l^{-1} agar, with a 24 h-old culture (Claus and Berkeley, 1986). The tubes were incubated at 4-55 °C, and examined after every seven days up to 21 days for the low temperatures (4-20 °C) and after 5 days for the higher temperatures (Guinebretière et al., 2008).

Production of haemolysin

A 24 h-old nutrient broth (HiMedia M002) culture was spotted on blood agar plate containing 50 ml defibrinated sheep blood l^{-1} blood agar base (HiMedia M834) and incubated for 16-18 h at 30 °C (Prüß et al., 1999). The results were expressed as ratio of clear zone diameter to diameter of the spot.

Production of Nhe enterotoxin

Enterotoxin production by the isolates was checked by using 3M Tecra Bacillus diarrhoeal enterotoxin visual immunoassay kit (3M Australia Pty Limited, Frenchs Forest, NSW, Australia). Brain heart infusion broth (HiMedia M210), supplemented with 10 g glucose l^{-1}, was inoculated with a 24 h-old culture, incubated at 37 °C for 24 h and centrifuged at 7830 g for 10 min. The supernatant was used for enterotoxin detection as per manufacturer's instructions. Amounts of produced enterotoxin were evaluated with index values derived from the Tecra reading scale; indices from 1 to 5 corresponded to the colouration intensity. According to the manufacturer's instructions, strains with an index of <3 were considered negative.

Results

Growth temperature requirement

Out of 144 isolates, 74% were able to grow at ≤7 °C and 15% at 20-50 °C (Table 1). Majority of milk (83%) and cheese (89%) isolates and all the isolates from ice cream, paneer and butter were able to grow at ≤7 °C.

Production of haemolysin

Out of 144 isolates, 93% exhibited β-haemolysis on sheep blood agar and showed a discontinuous haemolytic pattern (Fig. 1), characteristic for Hbl (Table 2). While majority of the isolates from milk were weak haemolytic, the same from rest of the products were moderate haemolytic.

Production of Nhe enterotoxin

Production of diarrhoeal enterotoxin component, NheA, was measured using Tecra

Bacillus diarrhoeal enterotoxin visual immunoassay kit (Fig. 2). Out of 144 isolates, 97% were positive for the production of diarrhoeal enterotoxin (Table 3). While majority of the isolates from milk, ice cream, paneer, cheese and butter were strong producers (colouration index, >4-5) of NheA enterotoxin, only 32% of the same from milk powder were strong producers.

Discussion

For the growth of *B. cereus* isolates, different ranges of temperature were observed, indicating their wide diversity and ecotype. Majority (74%) of the strains were able to grow at refrigeration temperature (≤7 °C), the criterion for considering those as
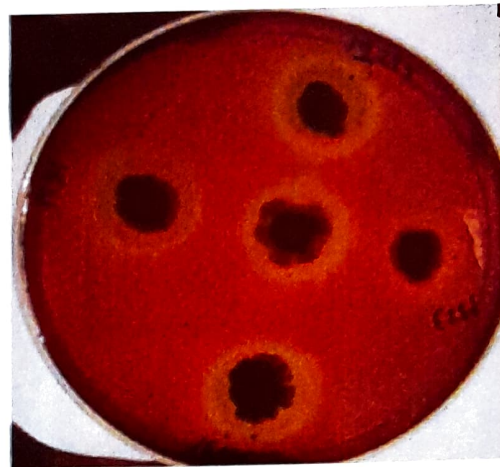

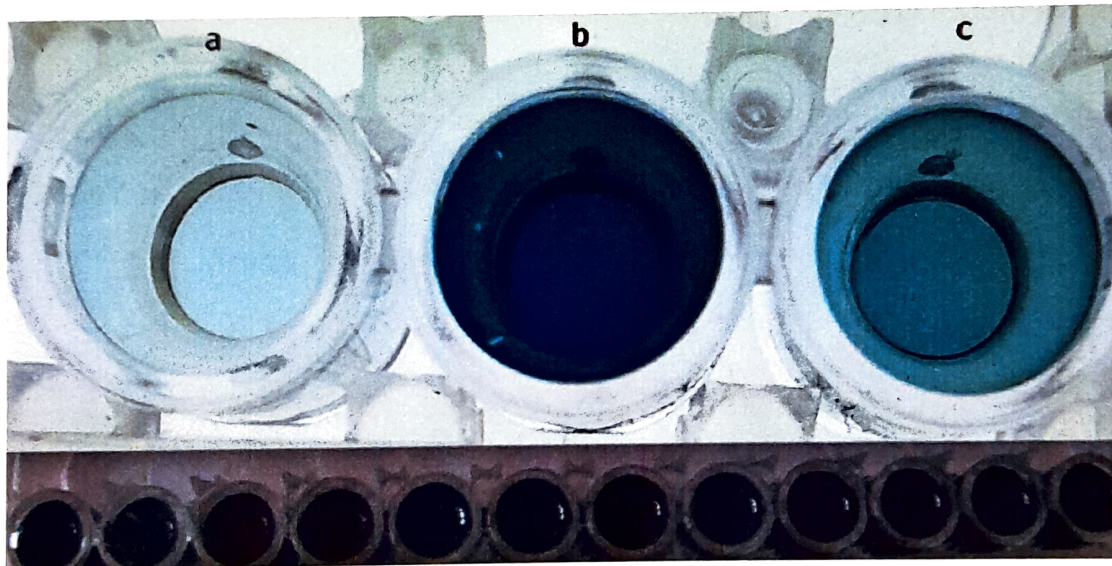**Fig. 1.** Haemolysis on sheep blood agar plate by some *Bacillus cereus* isolates.

Fig. 2. Diarrhoeal enterotoxin production by some *Bacillus cereus* isolates, detected by Tecra antibody test: a, negative control; b, test; c, positive control.

psychrotrophic (te Giffel *et al.*, 1995; Francis *et al.*, 1998). Presence of such a large number of psychrotropic strains in dairy environment is of major concern because of their potential for growth, spoilage and toxin production in chilled products, such as milk and dairy products (Anderson Borge *et al.*, 2001). Other studies also showed pasteurized milk and refrigerated food to frequently harbour psychrotrophic strains of *B. cereus* (te Giffel *et al.*, 1997; Svensson *et al.*, 2004). Thirty-four percent of the isolates from milk powder were moderate thermotolerant (20-50 °C). This may be attributed to adaptation or selection of thermotolerant strains during drying and

heating processes generally used to make milk powder.

Haemolysin is a three-component enterotoxin produced by *B. cereus*, which is one of the potential virulence factors in *B. cereus*-mediated diarrhoea (Beecher *et al.*, 1995). Ninety-three percent of the *B. cereus* isolates exhibited β-haemolysis, which was a discontinuous pattern in blood agar. This is as a result of a mutually inhibitory effect of B and L1 components and the slow reaction between the B component and the erythrocyte membrane (Stenfors Arnesen *et al.*, 2008). This is in consistence with the report of β-haemolytic activity exhibited by 92% of the *B.*

Table 1. Range of growth temperatures of *Bacillus cereus* isolates from milk and dairy products

Source	No. of Isolates	% of positive Isolates			
		4-40 °C	7-40 °C	10-45 °C	20-50 °C
Milk	83	71	12	5	12
Milk powder	32	31	0	34.6	34.4
Ice cream	11	90	10	0	0
Paneer	5	17	83	0	0
Butter	4	100	0	0	0
Cheese	9	89	0	11	0

Table 2. Clustering of 144 isolates of *Bacillus cereus* from milk and dairy products on the basis of haemolysin production ability

Group*	% of isolates					
	Milk	Milk powder	Ice cream	Paneer	Cheese	Butter
Non-haemolytic	10	16	10	33	0	0
Weak haemolytic	71	19	27	27	56	50
Moderate haemolytic	11	41	45	40	44	50
Strong haemolytic	8	24	18	0	0	0

*Isolates were designated as non-haemolytic (1), weak (>1-1.5), moderate (>1.5-2) and strong (>2) haemolysin producers, according to ratio of diameter of clear zone to that of colony spot, developed on blood agar after incubation at 30 °C.

Table 3. Clustering of 144 isolates of *Bacillus cereus* from milk and dairy products on the basis of diarrhoeal enterotoxin production ability

Group*	% of isolates					
	Milk	Milk powder	Ice cream	Paneer	Cheese	Butter
Non-producer	2	0	0	0	11	0
Weak producer	38	68	45	0	0	0
Strong producer	60	32	55	100	89	100

*Isolates were designated as non-producers (<3), weak producers (3-4) and strong producers (>4-5). Indices from 1 to 5 (within parentheses) correspond to colouration intensity according to the manufacturer's instructions.

cereus isolates from food ingredients and products in Brazil (Chaves *et al.,* 2011).

All the isolates from milk powder, ice cream, paneer and butter produced diarrhoeal enterotoxin. However, 98% of the isolates from milk and 89% from cheese were found positive for diarrhoeal enterotoxin. Results are in consistence with the earlier reports, where 96% of the isolates from various food products and 74% of the isolates from dairy production chain were Nhe positive (Moravek *et al.,* 2006; Svensson *et al.,* 2007). Semi-quantitative production index indicated 58% isolates were high producers (index >4-5) of NheA. Prevalence of high producers of Nhe among the dairy isolates is of significance, as Moravek *et al.* (2006) found cytotoxicity on Vero cells to be dominated by Nhe, indicating a high diarrhoeic potential of the toxin. However, an in-depth investigation of the individual and synergistic effect of toxins in combination with other known virulent determinants and effect of various environmental factors needs to be further studied.

Conclusion

Majority of the *B. cereus* strains isolated from milk and dairy products were able to grow at refrigeration temperature and were potent producers of enterotoxin Hbl and Nhe. The prevalence of potent producers of enterotoxin among dairy isolates having ability to grow at refrigeration temperature indicates high risk and public health concern. Observations in the present study may be important when considering microbiological criteria for *B. cereus* in pasteurized milk and dairy products and possibly in other foods stored at low temperature.

Acknowledgments

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Research Article

Comparative analysis of antioxidant activities and phytochemical properties of some culinary herbs

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Abstract

The present work aimed to evaluate the antioxidant activities as well as phytochemical analysis of leaf extracts of some commonly used leafy spices such as *Murraya koenigii (Mk), Coriandrum sativum (Cs), Trigonella foenum-graecum (Tfg)* and *Mentha* x *piperita (Mp)*. Lyophilised plant extracts (LPEs) were obtained by hot water extraction (HWE) process followed by rotavap and lyophilisation. Among the herbs tested, *Mk* showed the highest antioxidant activity in DPPH scavenging (77.35 % mg^{-1} of LPE), superoxide anion radical scavenging (60.21 % mg^{-1} of LPE) and hydrogen peroxide scavenging (57.21 % mg^{-1} of LPE) model. *Tfg* showed least activity in DPPH scavenging (33.15 % mg^{-1} of LPE) and superoxide anion radical scavenging (25.36 % mg^{-1} of LPE) assay while *Cs* had the least activity in hydrogen peroxide scavenging (43.70 % mg^{-1} of LPE) system. Phytochemical investigations revealed the presence of major primary and secondary metabolites. *Mk* possessed highest amount of phenolics (5.70 mg GAE g^{-1} of LPE), soluble sugars (68.18 mg GLE g^{-1} of FTW) and proteins (69.84 mg BSAE g^{-1} of FTW) and plant pigments (total chlorophyll 6.22 mg g^{-1} of FTW and total carotenoid 0.19 µg g^{-1} of FTW) among the herbs. SDS-PAGE and HPLC finger printing had been performed for analysis of protein patterns and phenolic compounds respectively. In conclusion, addition of culinary herbs and leafy spices that show high to moderate antioxidant activity with an excellent amount of phytochemicals in dietary items would go a long way in assuring human health and wellness as well as enhancement of the disease fighting capacity against oxidative stress related disorders.

Key words: Culinary herbs, Leafy spices, Antioxidant activity, SDS-PAGE, HPLC, Phytochemicals, Human health, Oxidative stress related disorders.

Introduction

Currently, one of the most numerously pronounced keyword related to food-health-disease concept is ROS i.e., Reactive Oxygen Species. ROS chemically includes all those oxygenated free radical (OFR) species exemplified as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$), peroxynitrite ($ONOO^{\bullet}$) as well as some non-radical forms (hydrogen peroxide, H_2O_2; hypochlorous acid, HOCl), frequently generated in biological systems by endogenous or exogenous factors. ROS, surprisingly besides playing super roles in energy production, phagocytosis, regulation of cell growth and intercellular signalling, or synthesis of biologically important compounds (Halliwell, 1997), hammer the living systems when produced in excess, resulting in

oxidative stress and causing oxidation of cellular biomolecules *viz.* carbohydrates, proteins, lipids and nucleic acids; membrane damage, decreasing membrane fluidity. Such damage in turn leads to several ROS-mediated diseases or disorders such as cancer, acquired immunodeficiency syndrome, malaria, cardiovascular disease, stroke, gastric ulcer, diabetes, malignant tumours, rheumatic joint inflammation, arthritis, cataracts, Parkinson's and Alzheimer's disease, old-age symptoms and aging etc. (Halliwell and Gutteridge, 1984; Maxwell, 1995; Halliwell, 2000; Young and Woodside, 2001; Moskovitz *et al.*, 2002; Heinecke, 2003). Scientific reports advocate that these diseases or disorders can be controlled or cured by regular intake of dietary antioxidants (Atoui *et al.*, 2005; Alasalvar *et al.*, 2005) as antioxidant molecules scavenge free radicals by inhibiting initiation and breaking chain propagation or suppressing the formation of free radicals by binding to the metal ions, quenching hydrogen peroxide and

superoxide and singlet oxygen (Shi *et al.*, 2001). Natural antioxidants present in dietary food-adjuncts or foodstuffs have only recently attracted attention because of their presumed safety concern as well as high therapeutic attributes. Different parts and products of plant such as fruits, vegetables, culinary herbs and spice etc. contain a wide variety of phytochemicals and provide a storehouse of natural antioxidants. Phytochemicals such as carotenoids, phenolic compounds, flavonoids, terpenoids, alkaloids, nitrogen compounds, vitamins, and some enzymes show remarkable antioxidant potentiality.

In most of the contemporary cuisine culinary herbs and leafy spices have been employed to impart diverse flavor, aroma, color and taste to various foods and drinks around the world. Foods and drinks in combination with culinary herbs are full of pharmacological agents; they act as drugs in the body and strengthen body's defense system. Ineffective defense system may lead to a high-risk of various disease developments. Majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic conditions in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of sedentary lifestyle, or due to the poor scavenging system in the body caused by the depletion of the dietary antioxidants (Schulz *et al.*, 2000; Dringen, 2000). In diet-based therapies, research investigations have been carried out to evaluate the medicinal importance of various culinary herbs and leafy spices. Hence the present study attempts to assess the healing power of culinary herbs and leafy spices in terms of antioxidant potentiality and explore their use in daily lives for the benefits of human health and management of diseases.

Materials and methods

Plant materials and sample collection

Fresh leaves or aerial parts of curry tree, coriander, fenugreek and peppermint were collected from local vegetable super market, Shivmandir (26°42'29.63" N and 88°21'40.52" E) Siliguri, Darjeeling. Plant specimens were identified and authenticated taxonomically by Prof. A.P. Das, Department of Botany, University of North Bengal, Siliguri, WB and Herbarium specimens were prepared (Table 1).

Extraction and preparation of lyophilized extract

For HWE, a modified method after Aliakbarlu and Tajik (2012) was followed. A 10 g of freshly washed and finely chopped leaf samples was extracted for 30 min under darkness by refluxing with HPLC grade deoxygenated water (1:10, w/v) at 100 °C in a temperature controlled water bath shaker with gentle agitation. After cooling, each sample was filtered through Whatman filter paper (Grade 1) and the solid residues obtained were further treated with same procedure twice. The filtrate fractions from every extraction process were pooled and concentrated under reduced pressure at 40 °C in a rotary evaporator equipped with chiller, followed by lyophilisation in a vacuum freeze-dryer to obtain the lyophilized crude extracts. The lyophilised extracts were weighed and re-dissolved in same fluid to prepare stock solutions of desired concentrations and subsequently stored in air tight vials at -20°C until use for analyses.

Determination of water soluble extractive value

The water soluble extractive (WSE) value was expressed in percentage (%) and was determined using the formula: % WSE = ($Weight_{lyophilised\ crude\ extract}$ / $Weight_{initial\ plant\ material}$) X 100. Water-soluble extractive value plays an important role in evaluation of crude plant extracts. It was observed that highest WSE value was in *M. koenigii* (7.73 %), followed by *M. piperita* (5.75 %) and *T. foenum-graecum* (4.96 %), and lowest in *C. sativum* (2.05 %).

Determination of total moisture content

Moisture content of leaf samples was determined using a laboratory oven kept at 105 ± 3°C for 24 h. The moisture content (%) was calculated according to AOAC (1975), using the following formula: Total moisture

Table 1. List of the herbs used in the present study along with their reported uses

English common name	Bengali vernacular name	Scientific name	Taxonomic family	Culinary use	Medicinal use
Curry tree	Kari pata	*Murraya koenigii* (L.) Spreng.	Rutaceae	As spice in different food preparations, curry powder, pickle, chutney, sausages and seasonings.	For curing dysentery, vomiting; essential oil from leaves exhibited a strong antibacterial and antifungal activity.
Coriander	Dhonay	*Coriandrum sativum* L.	Apiaceae	As seasoning in curries, salads and soup; a garnish on cooked dishes; in gravies and as green curry paste.	An appetite stimulant; leaves have antibacterial and antifungal properties; useful for headaches, muscle pain, stiffness and arthritis.
Fenugreek	Methi	*Trigonella foenum-graecum* L.	Fabaceae	In spicy soups and stews; as garnish; seasoning in curries, salads and sauses; microgreens used in salads.	Leaf showed anticholesterolemic, anti-inflammatory, antitumor, carminative, expectorant, febrifuge, hypoglycaemic, parasiticide.
Peppermint	Pudina	*Mentha* x *piperita* L.	Lamiaceae	In foods and drinks preparation, confectioneries and sweet liquors, sauces and salads; as garnish and stuffing.	Pain reliever, stimulating, stomachic, carminative, anti-spasmodic, treatment of cholera and diarrhoea.

content (%) = [(Weight$_{initial}$ - Weight$_{final}$)/ Weight$_{initial}$] x 100. The experiment was performed in triplicates ($n = 3$).

Analysis of antioxidant activities

Determination of total polyphenol content

The total polyphenol content was assayed spectrophotometrically at λ_{750} with FCR using gallic acid as the standard (Taga *et al.*, 1984). The total polyphenol content (TPC) was calculated as gallic acid equivalents (GAE) from a calibration curve of gallic acid standard solutions and expressed as mg of GAE g^{-1} of LPE. The experiment was performed in triplicates ($n = 3$).

Determination of total flavonoid content

Total flavonoid content was estimated spectrophotometrically at λ_{420} using the method described by Ordon ez *et al.* (2006). Total flavonoid content (TFC) was calculated as catechin equivalents (CAE) from a calibration curve of (+)-catechin standard solutions and expressed as mg of CAE g^{-1} of LPE. The experiment was performed in triplicates ($n = 3$).

DPPH' free radical scavenging activity

The DPPH• scavenging activity was monitored at λ_{517} using the method of Yen & Duh (1994), with slight changes. Free radical scavenging (FRS) activity expressed as percentage

inhibition (% I) of the DPPH• radical was calculated according to the formula given by Viuda-Martos *et al.* (2010): FRS activity (% I) = $[(A_c - A_s)/A_c] \times 100$, where A_c refers to the absorbance of control (t = 0 min) and A_s is the absorbance of sample plus DPPH• (t = 30 min). The experiment was performed in triplicates ($n = 3$).

Superoxide anion scavenging activity

The superoxide anion radicals ($O_2^{·-}$) scavenging activity was determined according to the method described by Nishikimi *et al.* (1972). Percentage of $O_2^{·-}$ scavenged at λ_{560} was measured using the formula: Superoxide anion scavenging (SAS) activity (% I) = $[(A_0 - A_s)/A_0] \times 100$, where A_0 was the absorbance of control, and A_s was the absorbance of sample extract at λ_{560}. The experiment was performed in triplicates ($n = 3$).

Hydrogen peroxide scavenging activity

The hydrogen peroxide (H_2O_2) scavenging activity was carried out following the procedure of Ruch *et al.* (1989). The percentage of H_2O_2 scavenging at λ_{230} by the extracts and standard were calculated using the following equation: H_2O_2 scavenging (HPS) activity (%) = $[(A_c - A_s) / A_c] \times 100$, where A_c was the absorbance of control and A_s was the absorbance of test sample at λ_{230}. The experiment was performed in triplicates ($n = 3$).

Phytochemical screening of extracts

Qualitative analysis of phytochemicals

Phytochemical screening to detect the presence or absence of some significant phytochemicals *viz.* phenols, flavonoids, tannins, alkaloids, cardiac glycosides, saponins, terpenes, steroid, etc. were performed according to standard methods (Harborne, 1973; Trease and Evans, 1989; Sofowora, 1993). The tests were based on the visual observation of colour change, chomophor formation or formation of a precipitation after addition of specific reagents or treatments.

Quantitative analysis of phytochemicals

Estimation of total soluble sugar content

For the estimation of total soluble sugar content, Anthrone's method as described by Plummer (1978) was followed. Total soluble sugar (TSS) content was calculated from a D-glucose calibration curve and results were expressed as mg of glucose equivalents (GLE) g^{-1} of fresh tissue weight (FTW). The experiment was performed in triplicates ($n = 3$).

Estimation of total soluble protein content

Total soluble protein was extracted using the standard protocol given by Chakraborty *et al.* (1995) and quantification was done according to Lowry *et al.* (1951) using BSA as standard. Total soluble protein (TSP) content was calculated as BSA equivalents (BSAE) from a calibration curve of BSA and expressed as mg of BSAE g^{-1} of fresh tissue weight (FTW). The experiment was performed in triplicates ($n = 3$).

Estimation of total carotenoid content

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). Absorbances of the sample were observed spectrophotometrically at λ_{645}, λ_{663} and λ_{480} and the total carotenoid content (TCR) was calculated by using the formula: TCR = $A_{480} - (0.114 A_{663} - 0.638 A_{645})$ µg g^{-1} fresh tissue weight (FTW). The experiment was performed in triplicates ($n = 3$).

Estimation of total chlorophyll content

Chlorophyll was extracted according to the method of Harbone (1998). Total chlorophyll content was estimated by observing the absorbance at 645 nm and 663 nm and calculated by the formula: total chlorophyll content (TCL) = $(20.2 A_{645} + 8.02 A_{663})$ mg g^{-1} fresh tissue weight (FTW). The experiment was performed in triplicates ($n = 3$).

SDS-PAGE analysis for protein pattern

To analyze the protein patterns of the samples, SDS-PAGE was performed on 10% resolving gels, as described by Sambrook *et al.* (1989).

HPLC fingerprint analysis for Phenolic compounds

For HPLC fingerprint analysis of phenolic compounds present in leaf extracts, a Shimadzu system (Shimadzu Corp., Kyoto Japan) was used. A flow rate of 1 ml min^{-1}, and gradient elution of acetonitrile-water-acetic acid (10:86:4, v/v/v) (solvent A) and of acetonitrile-water-acetic acid (80:16:4, v/v/v) (solvent B). 0-50 min solvent B from 0-100%; and injection volume of 20 µl were applied; whereas the separation of compounds was monitored at 280 nm and 320 nm (Pari and Latha, 2004).

Table 2. Qualitative detection of phytochemicals

Phytochemical category	*M. koenigii*	*C. sativum*	*T. foenum-graecum*	*M. piperita*
Reducing sugars	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	-	+
Alkaloides	+	+	+	-
Cardiac glycosides	+	+	+	+
Saponins	-	-	+	+
Terpenes	-	-	-	-
Steroids	+	+	+	-
Anthraquinones	+	-	+	-
Vitamin C	+	+	+	+

+ = present, - = absent

Statistical analysis of data

Experimental analyses were carried out in triplicate (n=3) and data ware expressed as mean ± standard deviation (SD). Statistical analysis was carried out by SPSS software (IBM SPSS, USA). One-way analysis of variance was performed by ANOVA procedures.

Result and Discussion

Because of their enchanting flavoral and toptastic qualities, culinary herbs and leafy spices have always been prized to Indian cuisine. Besides, these botanicals are an excellent source of versatile phytochemicals which have been reported to show good antioxidant activity. Natural antioxidants present in culinary herbs and leafy spices are responsible for inhibiting or preventing the deadly effects of oxidative stress. Herbs and spices contain free radical scavengers like polyphenols, phenolic acids and flavonoids. Polyphenols form a complex group of molecules associated with the cell walls of most plant species. This group of compounds ranges from simple phenolic acids (e.g., caffeic acid) to high-molecular-weight tannins. Polyphenols have various applications, such as in the production of paints, paper, cosmetics, as tanning agents, and as natural colorants and preservatives in the food industry. In addition, some phenolic compounds are antibiotics and anti-diarrheal, antiulcer, and anti-inflammatory agents and can be used in the treatment of diseases such as hypertension, vascular fragility, allergies, and hypercholesterolemia (Bravo, 1998; Higdon and Frei, 2003).

Preliminary phytochemical study of the extracts from *M. koenigii, C. sativum, T. foenum-graecum* and *M. piperita* showed the presence of major plant secondary metabolites *viz.* reducing sugars, phenolic compounds, flavonoids, cardiac glycosides and vitamin C, etc. but tannins, alkaloids, saponins, steroids and anthraquinones occurred in some of them (Table 2). Such secondary metabolites contribute significantly towards the biological activities of plant extracts such as antioxidant, antimicrobial, hypoglycemic, antidiabetic, anti-inflammatory activities etc. (Negi *et al.*, 2011).

In vitro antioxidant activity, principally, can be determined by hydrogen atom transfer (HAT) method and single electron transfer (SET) or electron transfer (ET) method (Joon & Shibamoto, 2009). HAT based methods measure the ability of an antioxidant to scavenge free radical by hydrogen donation to form a stable compound. SET based methods detect the ability of the antioxidant to transfer one electron to reduce compound including metals, carbonyls and radicals (Prior *et al.*, 2005; Huang *et al.*, 2005). Superoxide anion scavenging (SAS) assay, hydrogen peroxide

scavenging (HPS) assay, etc. involve HAT method, and the assays of total polyphenolic content (TPC), total flavonoid content (TFC) etc. are of ET method, while DPPH• assay include both the method predominantly via SET method (Karadag *et al.*, 2009; Badarinath *et al.*, 2010). The relatively stable radical DPPH has been used widely for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, medicinal plants and fruit extracts and food materials (Purohit *et al.*, 2005). Superoxide anion is a weak oxidant that gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress. Superoxide anion scavenging activity is correlated to the total flavonoids (Thaipong *et al.*, 2006). Hydrogen Peroxide radical scavenging activity is correlated to the presence of phenolic compounds. Generally, extracts that contain a high amount of phenolic compounds exhibit high antioxidant activity.

Antioxidant activity in terms of scavenging potentiality of hot water extracts of *M. koenigii, C. sativum, T. foenum-graecum* and *M. piperita* have been evaluated. All of them could act as potential radical scavengers in a concentration oriented fashion (Fig. 1). Interestingly *M. koenigii* showed the highest antioxidant activities scoring 77.354 % mg^{-1} of LPE in DPPH scavenging, 60.205 % mg^{-1} of LPE in superoxide anion radical scavenging and 57.209 % mg^{-1} of LPE in hydrogen peroxide scavenging assay, followed by *M. piperita*. Extract from *T. foenum-graecum* showed least scavenging activity in DPPH scavenging (33.145 % mg^{-1} of LPE) and superoxide anion radical scavenging (25.364 % mg^{-1} of LPE) system while *C. sativum* had the least activity in hydrogen peroxide scavenging assay (43.695 % mg^{-1} of LPE) .

The quantitative estimation of total moisture content, total soluble sugar, total soluble protein contents, total chlorophyll content, total carotenoid content present in the herb samples have been depicted in Table 3. Total moisture content among the herb samples was found in a range of 89% to 70%.The total soluble sugar content of was found highest in *M. koenigii* (68.18 mg GLE g$^-$

^{1} of FTW) and lowest in *T. foenum-graecum* (53.17 mg GLE g^{-1} of FTW), while content of
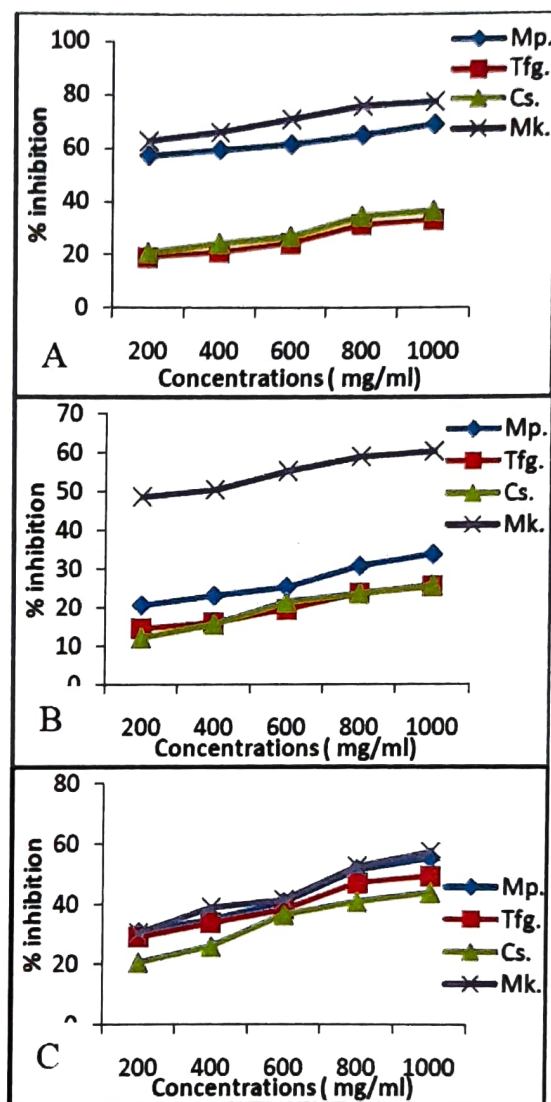

Fig. 1. Scavenging activity of the herb extracts. A. DPPH scavenging activity, B. Superoxide anion scavenging activity, and C. Hydrogen peroxide scavenging activity.

soluble protein was highest in *M. koenigii* (69.507 mg BSAE g^{-1} of FTW) and lowest in *C. sativum* (35.027 mg BSAE g^{-1} of FTW). Pigment analysis revealed that *M. koenigii* contained highest amount of total chlorophyll (6.223 mg g^{-1} of FTW) and carotenoid (0.190 μg g^{-1} of FTW) content. *T. foenum-graecum* and *M. piperita* showed the lowest chlorophyll content (1.639 mg g^{-1} of FTW) and lowest carotenoid content (0.063 μg g^{-1} of FTW) respectively. Garg *et al.*, 2012 also reported that chlorophyll content was higher in curry

leaves due to the darker shade of green, then the coriander.

Chlorophyll has been suggested as an effective antioxidant since it scavenges free radicals such as 1, 1-diphenyl-2-picrylhydrazyl (Khalaf *et al.*, 2008). Carotenoids that include xanthophylls and carotenes have the ability to detoxify various forms of activated oxygen and triplet chlorophyll that are produced as a result of excitation of the photosynthetic complexes by light. Dietary carotenoids are thought to provide health benefits due to their role as antioxidant molecules. SDS-PAGE analysis of proteins from the four herbs revealed the presence of a large number of bands in all cases, but not much difference was obtained among the herbs (Fig. 2).

The study also revealed that among the herbs *M. koenigii* ranked highest and *C. sativum* was lowest in total phenolic content and total flavonoid content (Table 4).Total

phenolic content and total flavonoid content in extracts of *M. koenigii* was found as 5.70 mg GAE g^{-1} of LPE and 1.68 mg CAE g^{-1} of LPE. Leaf extract from *C. sativum* contained 2.55 mg GAE g^{-1} of LPE as total phenolic content and 0.66 mg CAE g^{-1} of LPE as total flavonoid content. HPLC analysis of the phenols from all four herbs was carried out and results are shown in fig. 3. Among the four, maximum number of peaks was obtained in *M.koenigii* and the least in *M.piperita*. The health benefit of phenolics is directly linked to their antioxidant potentiality. Phenolics act as effective antioxidants is mainly due to their redox properties, which allow them to behave as reducing agents, hydrogen donors, and singlet oxygen quenchers. The potential hazard from oxidative stress in the body may be compensated through the consumption of a diet exclusively rich in antioxidant phenolics including polyphenols, phenolic acids and

Table 3. Nutritional and pigment contents of the herbs

List of herbs	Total moisture content[a]	Total soluble sugar content[b]	Total soluble protein content[c]	Total chlorophyll content[d]	Total carotenoid content[e]
M. koenigii	78.83 ± 0.16	68.18 ± 0.87	69.84 ± 0.39	6.22 ± 0.37	0.19 ± 0.01
C. sativum	82.11 ± 0.67	62.55 ± 0.65	35.00 ± 0.45	2.336 ± 0.10	0.12 ± 0.00
T. foenum-graecum	70.41 ± 0.72	54.17 ± 0.57	35.03 ± 0.53	1.639 ± 0.09	0.07 ± 0.00
M. piperita	89.56 ± 0.50	56.42 ± 0.07	45.53 ± 0.61	2.040 ± 0.02	0.06 ± 0.00

Content expressed as mean ± SD in a. percentage, b. mg GLE g^{-1} of FTW, c. mg BSAE g^{-1} of FTW, d. mg g^{-1} of FTW, e. µg g^{-1} of FTW. Fresh tissue weight is abbreviated as FTW.
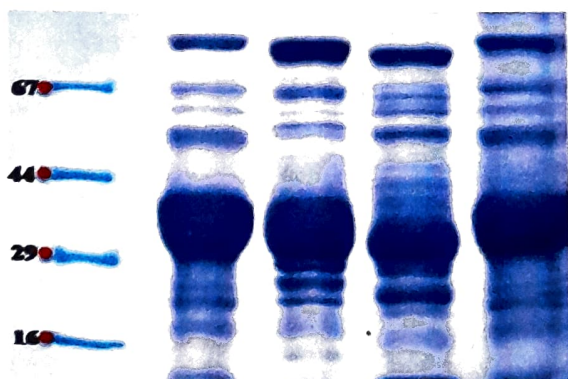

Fig. 2. SDS-PAGE analysis of protein of the herbs. Lane 1- Molecular weight markers in KDa; 2 - Tfg; 3- Mp; 4-Cs;5-Mk

Table 4. Total phenol and flavonoid contents[#].

Herb samples	TPC	TFC
M. koenigii	5.70 ± 0.20	1.68 ± 0.26
C. sativum	2.55 ± 0.05	0.66 ± 0.12
T. foenum-graecum	3.45 ± 0.10	1.33 ± 0.18
M. piperita	5.06 ± 0.01	1.75 ± 0.18

[#]Content expressed as mean ± SD, TPC as mg GAE g^{-1} of LPE and TFC as mg CAE g^{-1} of LPE.
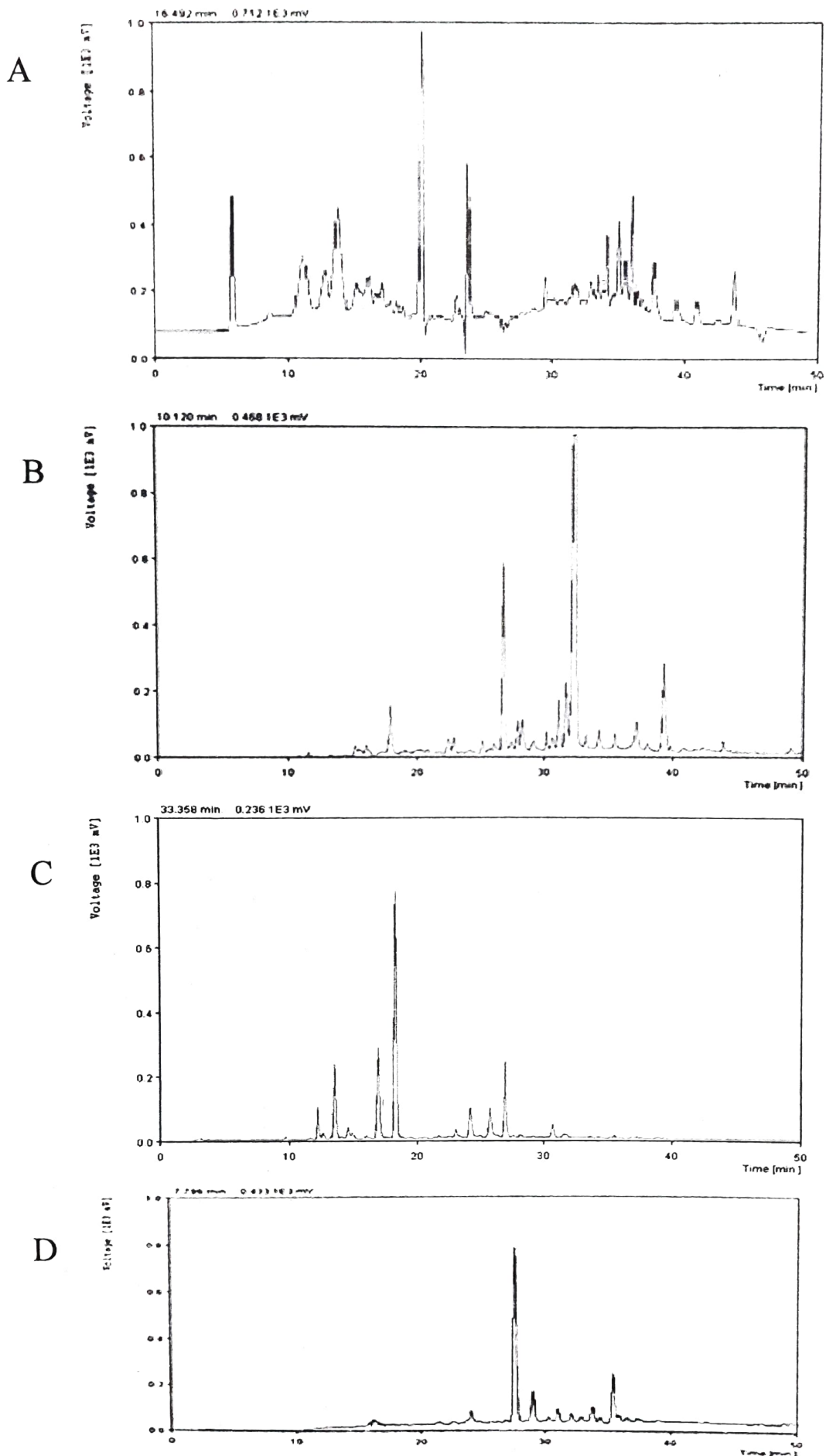
Fig. 3. HPLC chromatogram of phenolic compounds of herb extracts. A. *M. koenigii* B. *C. sativum* , C. *T. foenum-graecum* and D. *M. piperita*.

flavonoids. According to Scalbert and Williamson (2000) the amount of total human intake of phenolic compounds is about 1 g day^{-1} consisting two-thirds of flavonoids and one-thirds of phenolic acids.

Conclusion

Recently much attention has been focused on the analysis of dietary foods and food components. Food beyond its basic nutritional values has played some functional effects on prevention of diseases and maintenance of good health. Functional foods as the concept "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Thomas and Earl, 1994) are the centre of attraction for searching newer well-accepted functional foods from culinary herbs and leafy spices. As culinary herbs and leafy spices are full of variety in bioactive phytochemicals including antioxidant molecules and nutricomponents they can be incorporated as functional foods in our everyday's diets to rejuvenate ourselves.

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Research Article
Prevalence of begomoviruses associated with tomato leaf curl disease in the sub-Himalayan plains of West Bengal

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Abstract

Tomato is a solanaceous crop and one of the most economically important vegetables in the world. India ranks second in total production of tomato in the world. It has been referred to as a "functional food," a food that goes beyond providing just basic nutrition. ToLCD is one of the major constraints to tomato production in India. To study the disease incidence of tomato, a survey was made in the tomato crop growing fields of Darjeeling, Jalpaiguri, Coochbehar and Uttar Dinajpur districts of sub-Himalayan West Bengal during December 2015 to February 2016 and several infected and healthy leaf samples were collected based on the morphological symptoms like-vein clearing, leaf curling, leaf deformation and stunted growth of plants. Disease incidence ranged from 70% to 86.66% of the collected samples from different districts. All the samples collected from the present study area were tested by PCR with DengA and DengB primer and an expected amplicon of ~530bp was found. Two randomly selected PCR positive samples were sequenced and analyzed (Acc. Nos. KX108859 and KX108860). The SLG-1 isolate (Acc. No. KX108859) showed 95% nt identity with ToLCKV (Acc. No. KP178730) and the ISL-1 isolate (Acc. No. KX108860) showed 96% nt identity with ToLCNDV (Acc. No. KC513822). The threat of begomoviral spread to the north-eastern part of India has been taken into consideration.

Keywords: Solanaceous crop, Tomato leaf curl disease, *Begomovirus*, Coat protein.

Introduction

Tomato (*Lycopersicon esculentum* L.) is a solanaceous crop and one of the most economically important vegetables in the world (Hanssen *et al.*, 2010). India ranks second in total production of tomato in the world. In 2014, the production of tomato in India was 1,94,02,000 metric tons (1 t = 1000 kg) produced in a total area of 12,04,000 ha (1 ha = 10000 m^2), with an mean yield of 16.1 mt/ha(Indian Horticulture Database-2014). West Bengal stands eighth in the production of tomato in India, contributing about 17% of total production in India. Jalpaiguri and Coochbehar districts are the major tomato producing areas of West Bengal (Indian Horticulture Database-2014). It has been referred to as a "functional food," a food that goes beyond providing just basic nutrition. This is due to lycopene, a beneficial phytochemical. Tomatoes also play a role in

preventing chronic diseases and deliver other health benefits (Batta, 2016).

Tomato leaf curl disease (ToLCD) is one of the major constraints to tomato production in India. ToLCD-associated begomovirus is a member of the family *Geminiviridae* and transmitted through whitefly (*Bemisia tabaci*) in a persistent circulative non-propagative manner (Czosnek *et al.*, 1988; Hong and Harrison, 1995; Rana *et al.*, 2016). *Tomato leaf curl virus* (ToLCV) is characterized by twinned particles consisting of a circular, single-stranded (ss) DNA-A genome (~2.7 kb) (Stanley, 1985). It is often associated with a DNA-B or alpha- and/or beta-satellite molecules for successful symptom development (Dean *et al.*, 2001; Sohrab *et al.*, 2016). In India, occurrence of ToLCD was first reported by Vasudeva and Samraj in 1948. Altogether forty two strains of ToLCV have been reported to cause serious damage in tomato production worldwide (Vasudeva and Samraj, 1948; Sastry and Singh, 1973; Saikia and Muniyappa, 1989;

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Reddy *et al.*, 2005; Kirthi *et al.*, 2002; Paximadis *et al.*, 2001; Ramappa *et al.*, 1998; Brown *et al.*, 2015). Occurrence of ToLCV was reported from several places of West Bengal (Reddy *et al.*, 2005; Saha *et al.*, 2013; Saha *et al.*, 2014). In this communication, PCR amplification, sequencing and diversity analysis of partial coat protein (CP) gene of ToLCV have been reported infecting tomato in sub-Himalayan West Bengal.

Materials and methods

Survey, disease incidence and collection of diseased Samples

To study the disease incidence of tomato, a survey was made in the tomato crop growing fields of Darjeeling, Jalpaiguri, Coochbehar and Uttar Dinajpur districts of sub-Himalayan West Bengal during December 2015 to February 2016 (Fig. 1A, 1B) and several infected and healthy leaf samples were collected based on the morphological symptoms. Disease incidence was estimated using the method of James and Teng (1979).

Disease Incidence (%)
$$= \frac{\text{Number of plants infected}}{\text{Number of total plants}} \times 100$$

DNA isolation and PCR

Total DNA were extracted from the infected and healthy leaves following the method of Haible *et al.* (2006). Polymerase chain reaction (PCR) was done using Deng universal primer pair (DengA/DengB) and amplicons were visualized in 1.2% (w/v) agarose gels under UV-transilluminator (Fig. 1C).

Cloning, sequencing and sequence analysis

The purified PCR products were cloned into pGEM-T vector following the method of Sambrook and Russel (2001) and the clones were sent to Chromous Biotech Pvt. Ltd. for sequencing. The nucleotide sequences were aligned using ClustalW (Thompson *et al.*, 1994). The nucleotide were compared with the corresponding sequences of other isolates of ToLCV deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) using the BLAST analysis (Altschul *et al.*, 1997). Sequence identity matrix was generated using SDT 1.2 (Muhire *et al.*, 2014) and a

phylogenetic tree was generated by neighbor-joining method and Kimura-2 parameter using MEGA 6 (Tamura *et al.*, 2013).

Results and discussion

Symptomatology

Disease infected plants showed typical begomoviral symptoms like- vein clearing, leaf curling, leaf deformation and stunted growth of plants. Disease incidence varies from location to location like 78.57% in Darjeeling, 86.66% in Uttar Dinajpur, 80% in Jalpaiguri and 70% in Coochbehar district. Similar types of symptoms were reported earlier by several workers (Padidam *et al.*, 1995; Saha *et al.*, 2014; Saha *et al.*, 2013).

Molecular characterization and phylogenetic analysis

All the samples collected from the present study area were tested by PCR using universal Deng primer pair. On PCR with DengA and DengB, all the infected samples gave an expected amplicon of ~530bp (Fig. 1D) but none of the healthy samples showed positive result. Two randomly selected PCR positive samples were sequenced and analyzed. Similar types of results were also described by Reddy *et al.* (2005), Briddon *et al.* (2008) and several other workers (Brown *et al.*, 2001; John *et al.*, 2006; Santoso *et al.*, 2008; Samad *et al.*, 2009; Haider *et al.*, 2007).

The partial CP (AV1), Pre-CP (AV2) region of the viruses were sequenced and submitted in the GenBank database (Acc. Nos. KX108859 and KX108860). When the nucleotide (nt) sequences of the isolates were compared with those of other *Begomovirus* available in the GenBank, the SLG-1 isolate (Acc. No. KX108859) showed 95% nt identity with *Tomato leaf curl Karnataka virus* (ToLCKV) (Acc. No. KP178730) and the ISL-1 isolate (Acc. No. KX108860) showed 96% nt identity with *Tomato leaf curl New Delhi virus* (ToLCNDV) (Acc. No. KC513822) as shown in the fig. 2A. In the phylogenetic tree, ToLCKV and ToLCNDV form different clusters with the other subsequent members of the respective viruses (Fig. 2B).

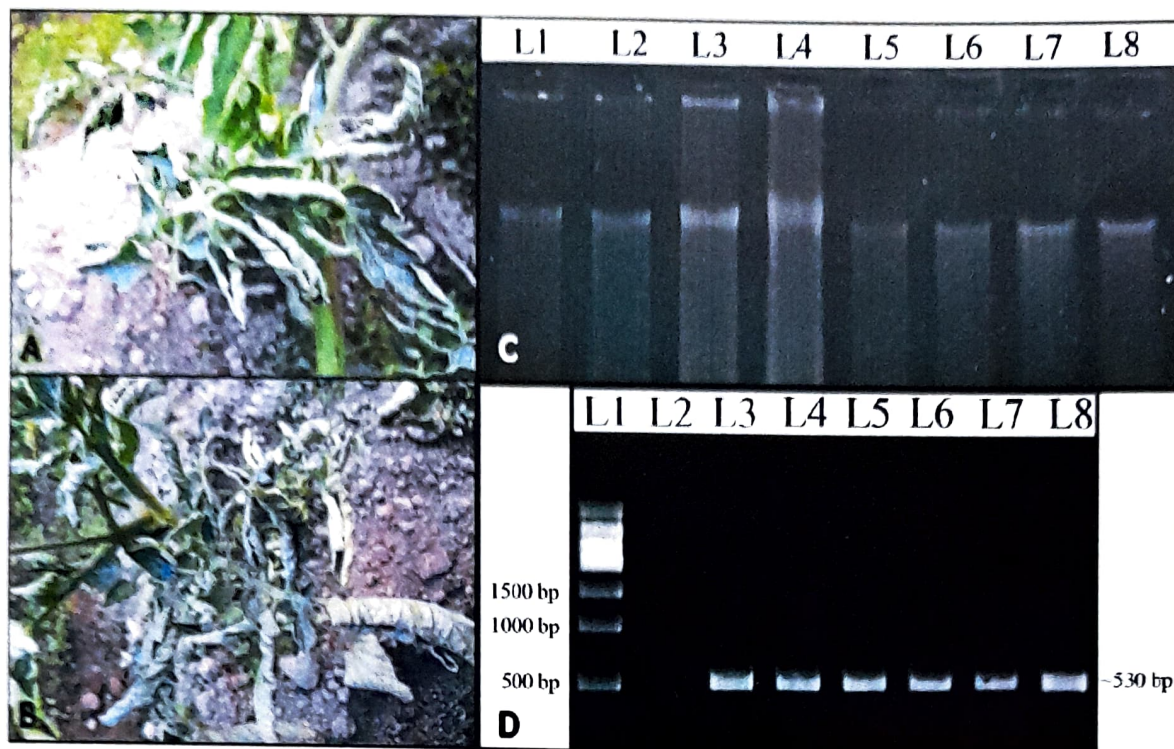As the number of strains of ToLCV has increased a lot, the whole genome sequencing

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Fig. 1. (A & B) Naturally infected tomato plants; (C) Total DNA on agarose gel isolated from healthy (L1-L3) and infected (L4-L8) plants; (D) Amplified PCR product on agarose gel isolated from healthy (L2) and infected (L3-L8) plants; L1- 500 bp DNA ladder.

Table 1. ToLCKV and ToLCNDV isolates used in the study along with their GenBank Acc. No., host and place of occurrence

| SL No.	Acc. No.	Organism	Place	Host	Country
1	KX108859**	ToLCKV	Siliguri	*Lycopersicon esculentum*	India
2	KP178730	ToLCKV	Maharashtra	*Lycopersicon esculentum*	India
3	KP178731	ToLCKV	Maharashtra	*Lycopersicon esculentum*	India
4	EU604297	ToLCKV	Lucknow	*Petunia* sp.	India
5	JX987088	ToLCKV	Lucknow	*Zinnia elegans*	India
6	AY375241	ToLCKV	Lucknow	*Lycopersicon esculentum*	India
7	KX219744	ToLCKV	Andhra Pradesh	*Helianthus annuus*	India
8	KF663699	ToLCKV	Punjab	*Lycopersicon esculentum*	India
9	KF551581	ToLCKV	Punjab	*Lycopersicon esculentum*	India
10	KP195261	ToLCKV	Punjab	*Lycopersicon esculentum*	India
11	AY754812	ToLCKV	Janti	*Lycopersicon esculentum*	India
12	AY753203	ToLCKV	Karnataka	*Lycopersicon esculentum*	India
13	FJ436982	ToLCKV	Bahraich	*Capsicum annum*	India
14	HM851186	ToLCKV	New Delhi	*Lycopersicon esculentum*	India
15	KX108860**	ToLCNDV	Islampur	*Lycopersicon esculentum*	India
16	KC545812	ToLCNDV	Delhi	*Cucumis sativus*	India
17	KM383743	ToLCNDV	Jamalpur	*Lycopersicon esculentum*	Bangladesh
18	KM383742	ToLCNDV	Jamalpur	*Lycopersicon esculentum*	Bangladesh
19	KM383741	ToLCNDV	jessore	*Lycopersicon esculentum*	Bangladesh
20	KM383739	ToLCNDV	Joydibpur	*Lycopersicon esculentum*	Bangladesh
21	KM383738	ToLCNDV	Joydibpur	*Lycopersicon esculentum*	Bangladesh
22	KM383737	ToLCNDV	Sylhet	*Lycopersicon esculentum*	Bangladesh
23	AF448058	ToLCNDV	Dargai	*Lycopersicon esculentum*	Pakistan
24	KC513822	ToLCNDV	Lucknow	*Lycopersicon esculentum*	India
25	AF448059	ToLCNDV	Islamabad	*Lycopersicon esculentum*	Pakistan

** Isolate under study
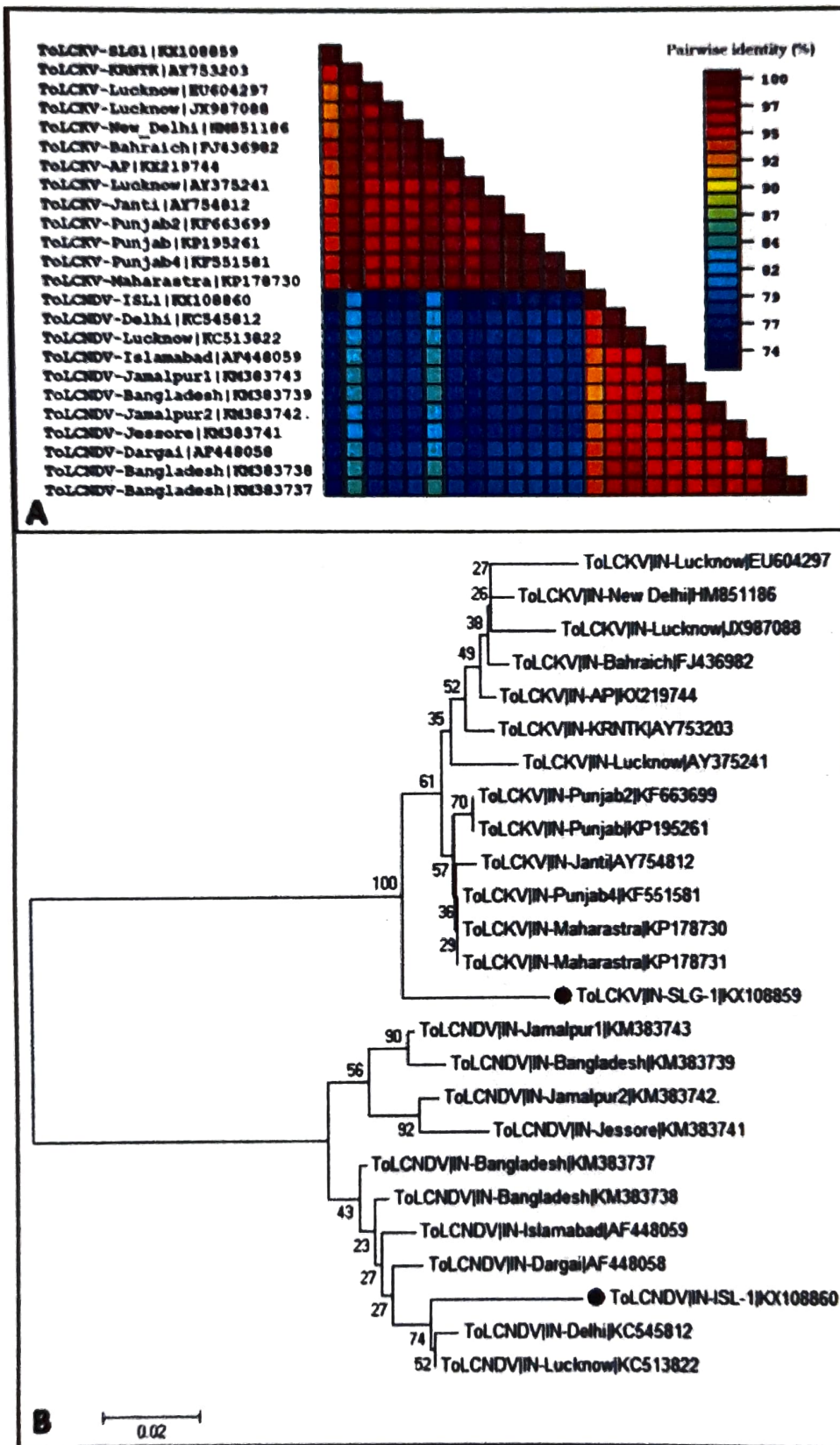
Fig. 2. (A) Sequence identity matrix of the 14 ToLCKV and 11 ToLCNDV isolates. Identity percent corresponds to the color matrix is indicated on the right side of the figure; (B) Phylogenetic tree generated by neighbour joining of ToLCKV- and ToLCNDV- CP alignments. Values at the nodes indicate percentage of bootstrap support (out of 1000 bootstrap replicates). GenBank accession numbers along with the collection spot of the viruses have been indicated at the end of each branch.

of the viruses are now-a-days essential for identifying different species as proposed by Kings *et al.* (2011). However, the ToLCV-CP gene analysis may provide valuable information to the recent occurrence of ToLCD in sub-Himalayan region of West Bengal. It can be said that, the high disease incidence may be attributed to prevalence of whitefly vector, warm tropical climate supporting year round survival of the whitefly, intensive cultivation of crops and polyphagous nature of the whitefly serving path of sustenance of begomovirus in alternative hosts (Saha *et al.*, 2014). The bipartite ToLCNDV has been reported to infect tomato and other solanaceous crops in the Indian subcontinent (Padidam *et al.*, 1995; John *et al.*, 2006; Santoso *et al.*, 2008; Saha et al, 2013). Although, ToLCKV was thought to be a recombinant strain and restricted to the southern part of India (Chatchawankanphanich and Maxwell, 2002). The threat of begomoviral spread to the north-eastern part of India has been taken into consideration and this may be correlated to different factors like- weather condition, tomato live stock import-export, and agricultural practices, that is operational in the study area.

Acknowledgement

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Research Article

Evaluation of streptomyces and non-streptomyces actinomycetes isolates for growth promotion in *Vigna radiata* and their use as biocontrol agent against *Sclerotium rolfsii*

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Abstract

Two streptomyces (ARHS/PO26 and ARHS/PO27)and two non streptomyces (ARHS/Mn3 and ARHS/Mn7) actinomycetes isolates obtained from the rhizosphere soil of *Solanum tuberosum* and *Mangifera indica* were found to be phosphate solubilizers and showed antagonistic activity against *Sclerotium rolfsii*. Isolates ARHS/PO26 and ARHS/PO27 were identified morphologically and confirmed by the National Centre for Fungal Taxonomy, as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08). ARHS/Mn 3 and *Streptomyces griseolus* (ARHS/PO27) could inhibit 68% and 59.7% growth of *Sclerotium rolfsii in vitro*. *In vivo* evaluation of the isolates ARHS/Mn 3, *Streptomyces griseolus* (ARHS/PO27) and *Streptomyces griseus* (ARHS/PO26) showed maximum growth promotion on *Vigna radiata* by enhancing key defense enzymes like chitinase, β-1,3-glucanase, phenylalanine ammonia lyase and peroxidase. The results emphasize the fact that soil actinomycetes could be used as potential biocontrol agents.

Keywords: Non-streptomyces Actinomycetes, *Streptomyces griseus*, *Streptomyces griseolus*, *Vigna radiata*, Growth promotion, Defense enzymes, *Sclerotium rolfsii*

Introduction

Mung bean or Green gram *Vigna radiata* (L.) Wilczek (syn: *Phaseolus aureus* Roxb.) constitutes the important group of grain legumes which form a major source of dietary proteins of high biological value, energy, minerals and vitamins (Taylor *et al.*, 2005). Those who can not eat animal protein this plant belonging to the family Fabaceae or leguminosae is a good source of protein. However, the yield of mung bean is greatly reduced due to various factors of which diseases caused by fungi and viruses are of major concern (Satya *et al.,* 2011). Now it has become necessary to find out ways of increasing yield and decreasing disease incidence in *Vigna*.

Streptomycetes are a group of actinobacteria which are part of the microbial flora of most natural substrates (Moustafa *et al.,* 1963) and mainly found in the rhizosphere of plants in association with other microorganisms like rhizobacteria and fungi. They utilize humic acid and other organic matter in soil. In their natural habitat, such as forests, the actinomycetes interact in various ways with the higher plants (Lo *et al.*, 2002). These organisms are part of PGPM or plant growth promoting microorganisms. Streptomycetes affect plant health in various ways like by producing plant growth promoting hormones like IAA (Manulis *et al.,* 1994), production of siderophores (Tokala *et al.,* 2002) which influence plant growth or by protecting the plant against plant pathogenic microorganisms. It has been reported that secondary metabolites produced by some *Streptomyces* spp. inhibit growth of phytopathogenic fungi like *Colletotrichum musae* and *Fusarium oxysporum* (Taechowisan *et al.,* 2005).Many of non-streptomycete actinomycetes (NSA) taxa are therefore rarely reported in literature dealing with routine isolations of biocontrol agents and plant growth promoters from plant and soil. Seed-coating with powder formulation of the biocontrol agent was as effective as drench application of the fungicide, oxine benzoate (No-Damp), in controlling *Rhizoctonia* damping-off and superior to the commercial biocontrol agent, *Streptomyces*

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griseoviridis (Mycostop), applied to tomato seeds as seed-coating. Ensign *et al.* (1993) reviewed the physiology of some NSA as a component of soil microflora. Although Lechevalier (1988) and Doumbou *et al.* (2001) reviewed the literature on the biological control of soil-borne fungal plant pathogens and plant growth promotion by actinomycetes, they covered activities mainly of *Streptomyces* spp.

The present study reflects the role of non-streptomyces strains- ARHS/Mn 3 and 7 and streptomyces strains (*Streptomyces griseus* and *S. griseolus*) as plant growth promoters and biocontrol agents in reducing sclerotial disease in *Vigna radiata*.

Material and Methods

Isolation of actinomycetes

Actinomycetes were isolated by the standard serial dilution plate technique by Warcup (1955) using starch casein nitrate agar (SCN) medium.

Biochemical Characterization

Biochemical characterization of actinomycetes isolates were performed including starch hydrolysis, catalase and indole tests.

Screening for phosphate solubilizing activity

Isolates were screened in Pikovskaya medium for phosphate solubilization activity (Pikovskaya, 1948). Isolates were inoculated in Pikovskaya media and incubated for 7 days. A halo zone around the growth indicates positive result for phosphate solubilization activity.

Selection of two non-Streptomyces and two Streptomyces actinomycetes strains

On the basis of *in vitro* plant growth promoting activities out of the isolates of actinomycetes, two non-streptomyces strains (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected. ARHS/PO26 and ARHS/PO27 were morphologically identified and confirmed by the National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08).

In vitro antagonistic effect on Sclerotium rolfsii

Streptomycetes and non-streptomycetes isolates were tested for antagonistic effect against *Sclerotium rolfsii* by dual culture method (Skidmore and Dickinson, 1976). Inhibitions of the radial growth of fungal pathogen by the isolates confirm antagonism.

Inoculation technique and disease assessment

15 days old plant (*Vigna radiata*) was used for artificial inoculation with fungal pathogen. Sand maize meal media containing fungal inoculum were added carefully in the rhizosphere and ensured that inocula were attached to healthy roots. Disease assessment was done 15 days after inoculation. In order to determine the effects of two non-streptomyces and two streptomyces strains on disease reduction, four treatments were taken in each case: untreated control; inoculated with pathogen; inoculation with test isolates and inoculation with both test isolate and fungal pathogen. Percentage disease incidence was recorded while disease intensity was calculated using a 0-6 scale (Mathew and Gupta, 1996).

Field Trial

Two non-streptomyces (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected for *in vivo* evaluation of the growth promoting activity on *Vigna radiata*. For seed coating the seeds were soaked in cell suspension overnight. For preparation of cell suspension 7 days old broth culture were centrifuged at 10000rpm for 10 min and the cell pellet was dissolved in 250ml sterile distilled water and tween-20. Growth measurement were observed 15 days after inoculation and dry biomass were measured after three months of inoculation.For growth promotion average root length, shoot length, total height, fresh weight and dry weight were measured against control.

Biochemical analyses

Leaves of *Vigna* plants treated with actinomycetes were used for all biochemical analyses. Leaves were collected for assay 15days after inoculation.

Enzyme assays

Peroxidase (POX. EC1.11.1.7.)

Extraction and assay of peroxidase was done following the method described by Chakraborty *et al* (1993). The plant tissues were macerated to powder in liquid nitrogen and extracted in 5 ml of chilled 0.05(M) sodium phosphate buffer (pH 6.8) containing 2 mM β-mercaptoethanol. One ml of 0.2(M) Na-phosphate buffer (pH 5.4), 1.7 ml dH$_2$O, 100µl crude enzyme, 100 µl O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H$_2$O$_2$ were used in the reaction mixture. Activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H$_2$O$_2$.

Chitinase (CHT, EC 3.2.1.14)

Chitinase was extracted and assayed from leaves following the method of Boller and Mauch (1988). 10µl of 1M Na-acetate buffer (pH 4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin were used in the reaction mixture. Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 rpm for 3 min. 0.3 ml supernatant, 30µl of 1M K-PO$_4$ buffer (pH 7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH 9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C. The amount of GlcNAc released was measured spectrophotometrically at 585 nm and activity was expressed as µg GlcNAc released /min/ g fresh wt. tissue.

Phenylalanine Ammonia Lyase (PAL, EC 4.3.1.5)

Enzyme was extracted and assay was done following the method described by Bhattacharya and Ward (1987). The assay mixture contained 500µl crude enzyme, 300µl of 0.3mM borate buffer (pH 8.0), 300µl of 2% L-phenylalanine and 1.9 ml distilled water. The mixture was allowed to incubate for 1 hr at 40°C and then absorbance value was measured at 290 nm. The enzyme activity was described as the amount of cinnamic acid produced from L-phenyl alanine by the enzyme from 1 g tissue/min.

β- 1,3-glucanase (β-GLU, EC 3.2.1.39)

β-1,3-glucanase was extracted and assayed from leaf samples following the method of Pan *et al.* (1991).The reaction mixture consisted of 62.5µl crude enzyme and 62.5µl 4% laminarin which was incubated at 40°C for 10 min and 375µl DNSA (dinitro salicylic acid) added to the mixture following incubation for 5 min on a boiling water bath. Finally the colored solution was diluted with 4.5 ml water and the amount of glucose liberated was determined spectrophotometrically. Activity was expressed as µg glucose released /min/g tissue.

Results and Discussion

Actinomycetes isolates were characterized by morphologically as well as biochemically. Out of the isolated isolates, two non-streptomyces and two streptomyces actinomycetes isolates showed positive result for biochemical tests.On the basis of *in vitro* plant growth promoting activities and phosphate solubilising activities *in vitro* two non-streptomyces strains (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected for further studies (Table 1, Fig. 1). ARHS/PO26 and ARHS/PO27 were identified and morphological identification were confirmed by the National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08).

Isolates were tested for *in vitro* antagonistic effect against *Sclerotium rolfsii*. ARHS/Mn 3 and *Streptomyces griseolus* (ARHS/PO27) were comparatively more effective to control *S. rolfsii*. ARHS/Mn3 and *S. griseolus* (ARHS/PO27) could inhibit 68 % and 59.7% growth of *Sclerotium rolfsii* (Table 2, Fig. 2). Results revealed that among the isolates tested, sclerotial blight disease development was markedly reduced with prior applications of isolates of *S. griseolus* (ARHS/PO 27) and ARHS/Mn 3 in comparison to *S. griseus* (ARHS/PO26) and ARHS/Mn7 (Table 3). Increase in the growth was observed in terms of increase in height of

Table 1: Biochemical tests of two non-streptomyces actinomycetes (NSA) and two streptomyces isolates

Isolates Code	Catalase test	Indole test	Starch hydrolysis	Phosphate solubilising activity
ARHS-Mn3	+	+	+	+
ARHS-Mn7	+	+	+	+
Streptomyces griseolus (ARHS/PO27)	+	+	+	+
Streptomyces griseus (ARHS/PO26)	+	+	+	+

Mn- Rhizosphere soil of *Mangifera indica* (25°32'12"N .88°24'45" E); PO- Rhizosphere soil of *Solanum tuberosum* (26°33.676'N 89°03.149'E).

Table 2: *In vitro* antagonistic activity of Actinomycetes isolates against *Sclerotium rolfsii*

Isolates	% of inhibition of *Sclerotium rolfsii*
ARHS-Mn 3	68.00
ARHS-Mn 7	56.00
Streptomyces griseolus (ARHS/PO27)	59.70
Streptomyces griseus (ARHS/PO26)	57.00

Table 3: Evaluation of isolates on the development of sclerotial blight incidence of *Vigna radiata*

Treatments	Disease Index[*] of *Vigna radiata*
S. rolfsii	6.35
S. rolfsii + *S. griseus* (A/RHS/Po26)	0.84
S. rolfsii + *S. griseus* (A/RHS/Po27)	0.81
S.rolfsii+ ARHS/MN 3	0.80
S.rolfsii+ ARHS/Mn 7	0.85

[*]0 = No symptoms; 1= Small roots turn rotten lesion appeared at the collar region; 2= Middle leaves start wilting and 10-20% of root turn brown; 3= Leaves wilted and 20-40% roots become dry with browning of shoot; 4= Extensive rotting at the collar region of roots, 60-70% of roots and leaves wilted, browning of shoot over 60%; 5= 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%; 6= Whole plants die. Average of 3 separate inoculated plants (15 days after inoculation)

Evaluation of selected isolates on the growth and development of *Vigna radiata* was conducted in *in vivo* conditions. Marked increase in attributes of parameters in growth of *V. radiate* was noticed when actinomycetes were applied in the rhizosphere of plants.

saplings, number of shoots and number of leaves and roots. Growth parameters were recorded from 15 days onwards (Fig. 3 and 4). Better growth enhancement was observed by *S. griseolus* (ARHS/PO27) in comparison to other streptomyces isolate. ARHS/Mn 3 also showed promoted better growth in mung bean in respect to control as well as ARHS/Mn 7 strain.

Activities of POX, PAL, chitinase and glucanase were also observed after application of bacterial strains. POX acitivities were increased more in ARHS/Mn 3 and ARHS/Mn 7 treated plants. In PAL acitivity, *Streptomyces griseolus* (ARHS/PO27)strain showed better results in comparison to other strains and control. Similarly, chitinase and glucanase activities were increased after application of ARHS/Mn 3 strain (Fig. 5).Induced systemic resistance (ISR) is effective against different types of pathogens but differs from systemic acquired resistance (SAR) in that the inducing PGPR does not cause visible symptoms on the host plant (Van Loon *et al.,* 1998). Pieterse *et al.* (2002) confirmed that to protect themselves from the disease, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often play crucial roles. The phenomenon of SAR suggests that there is a signal that originates at the site of elicitor (biotic or abiotic) application and moves throughout the plant. The activation of SAR turns the compatible plant-pathogen interaction into an incompatible (Uknes *et al.,* 1992) one. This resistance was correlated with the accumulation of pathogenesis related (PR) proteins, generally assumed to be markers of defense response (Ward *et al.,* 1991).

It can be concluded from the results of the present study that ARHS/Mn 3 and *S.*

griseolus (ARHS/Po27) can be used as good growth promoters as well as biocontrol agents against *S. rolfsii* in *Vigna radiata* in comparison to other selected non-streptomyces and streptomyces actinomycetes strains. Non-streptomyces actinomyces (NSA)
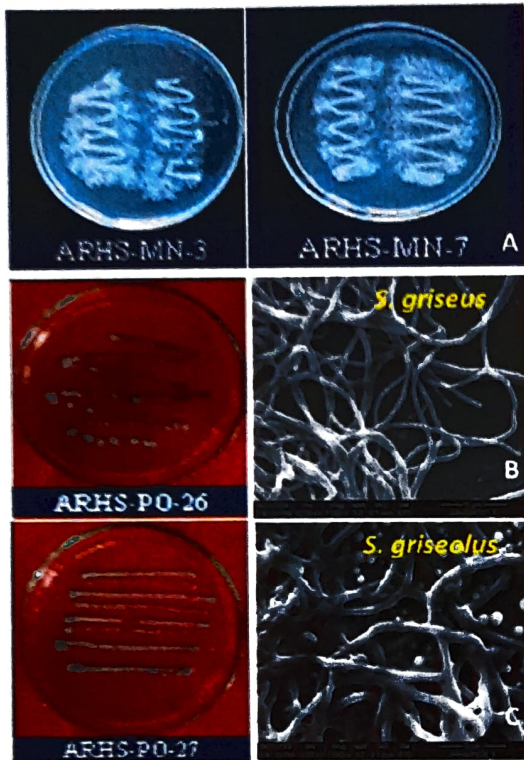

Fig. 1: Actinomycetes strains on starch casein nitrate agar (SCN) medium (A); Scanning electron microscopic view of *Streptomyces griseus* (ARHS/PO26) (B) and *Streptomyces griseolus* (ARHS/PO27) (C).
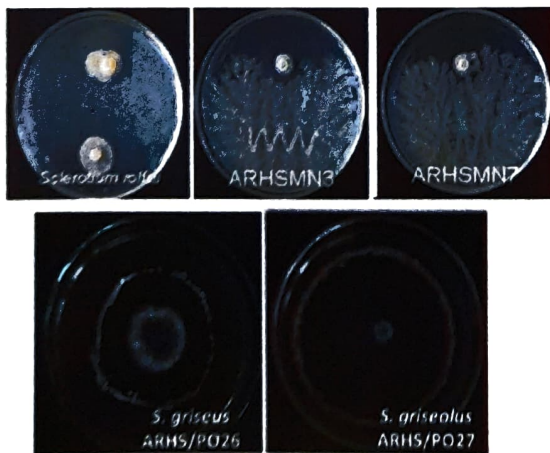

Fig. 2: Antagonistic activity of streptomyces and non-streptomyces actinomycetes isolates against *Sclerotium rolfsii*
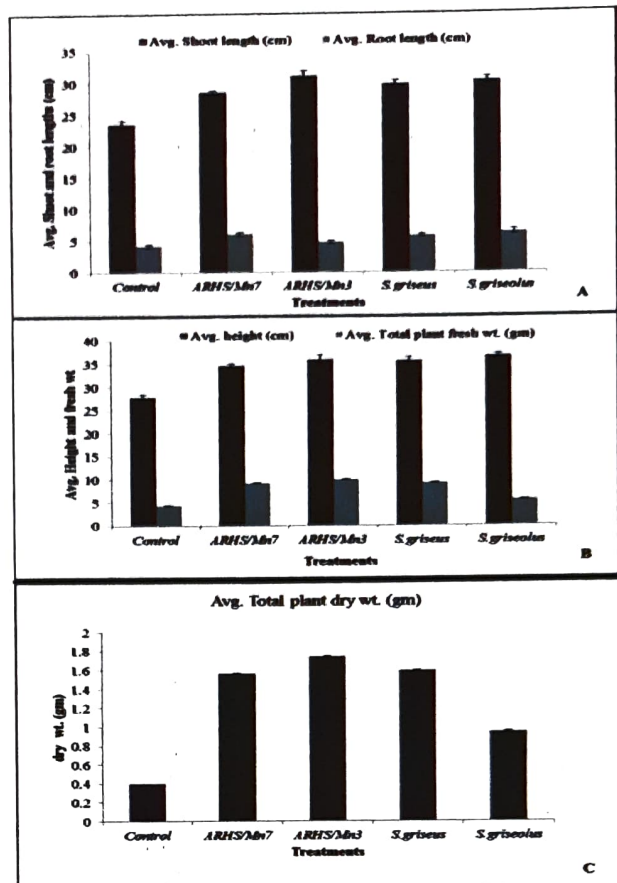

Fig. 3: Growth promotion in *Vigna radiata* after 15 days of treatment with streptomyces and non-streptomyces actinomycetes

Fig. 4: Growth enhancement of *Vigna radiata* after application of ARHS/Mn 3 strain (B) and *Streptomyces griseolus* (ARHS/PO27) (C) in comparison to control (A).

have great potential as candidates for the biocontrol of soil-borne fungal plant pathogens and also as plant growth promoters. With better understanding and screening of NSA, successful candidates from among NSA for biocontrol and plant growth promotion could be sourced.
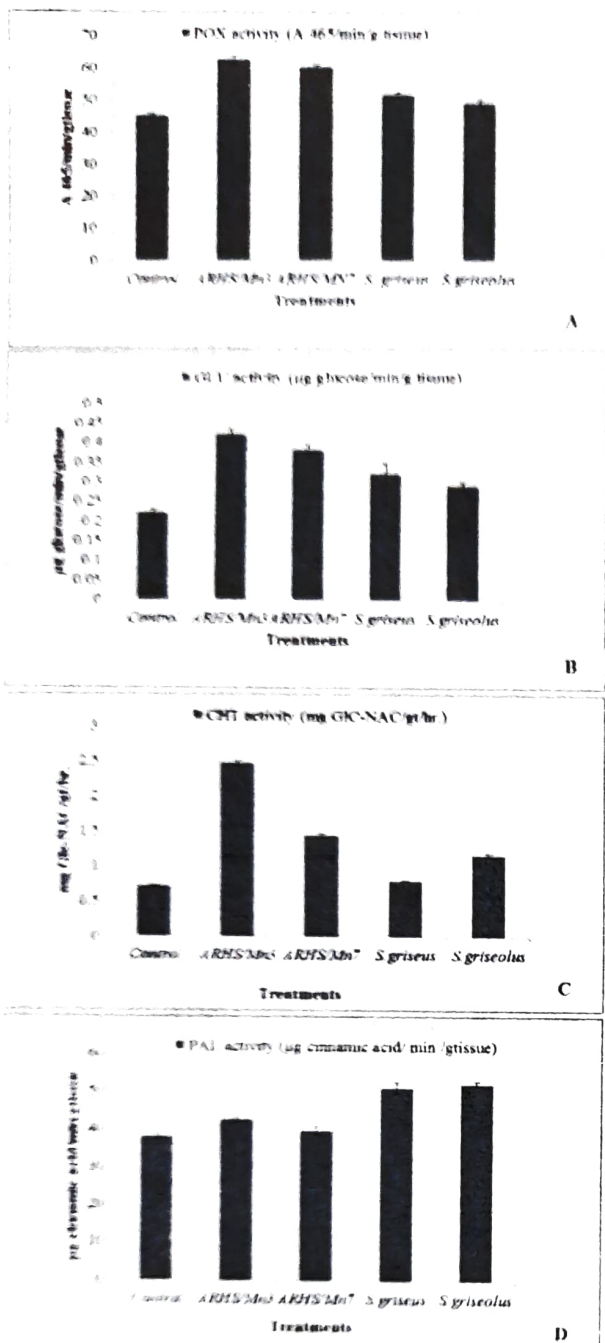
Fig. 5: Changes in defense enzyme activities in *Vigna radiata* after application of streptomyces and non-streptomyces actinomycetes isolates.

Acknowledgement

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