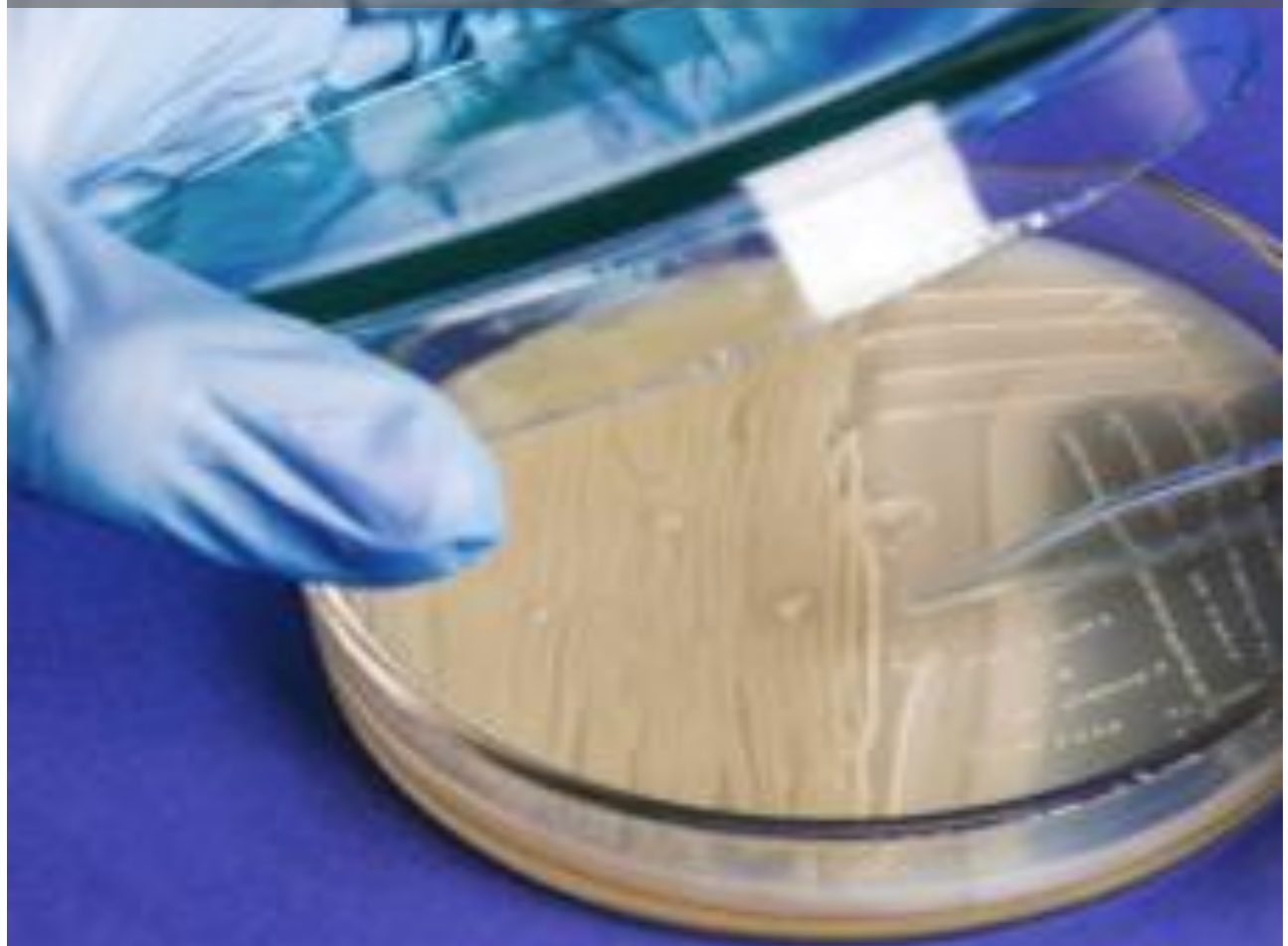


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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

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Review

## Microbial intervention in agriculture: An overview

Amrita Sengupta\* and Sunil Kumar Gunri

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With increase in population, rapid urbanization and industrialization, land area under agricultural production is decreasing day by day. In order to feed the huge population, more production is required from lesser area, which triggers continuous applications of higher doses of inorganic fertilizers in an injudicious manner posing serious harm on soil health, further rendering large fraction of land unfit for cultivation every year due to nutrient imbalance. Combustion of fossil fuels during production of inorganics, leaching, loss of excess inorganic nitrate and phosphorus from cropped lands, excessive uplifting of ground water for irrigation purpose also lead to degradation of the quality of environment and natural resources through global warming, eutrophication, heavy metal contamination in ground water, etc. Under such circumstances, some improvised technologies are to be adopted to enhance productivity in a sustainable manner. A great deal of effort focusing on the soil biological system and the agro-ecosystem as a whole is needed to enable better understanding of the complex processes and interactions governing the stability of agricultural lands. The technological advances made in recent times in exploring biodiversity have revealed that microbial diversity has immense potential that can be explored through careful selection of microbes and their successful utilization in solving major agricultural and environmental issues.

**Key words:** Agriculture, biological nitrogen fixation (BNF), plant growth promoting rhizobacteria (PGPR), phosphate solubilizing microorganisms, vesicular arbuscular mycorrhizae (VAM), arsenic detoxification.

### INTRODUCTION

The soil rhizosphere is a huge reservoir of microbial diversity. Microbes perform numerous metabolic functions essential for their own maintenance and can benefit the biosphere directly or indirectly through nutrient recycling, environmental detoxification, soil health improvement, waste water treatment, etc. A large fraction of beneficial soil microorganisms are still undiscovered and their ecological functions are quite unknown. Therefore, vast assays of microbial activities are the basic steps towards development of new technologies for

efficient utilization of microorganisms for attainment of sustainability in agriculture.

The greatest threats of the twenty-first century have become quite clear in the last few years. Climate change due to the vast increase in the production of greenhouse gases is real (Crowley, 2000). There is a genuine need for renewable energy supplies (Cook et al., 1991; Jackson, 1999). The diverse community of microorganisms constitutes "a metagenome of knowledge". This metagenome also extends to the microbial communities

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both inside and out of our body (Ahmad et al., 2011). Thus, microbial intervention in combination with developments in electronics, digital imaging and nanotechnology may play a major role in solving global challenges of the twenty first century including climate change.

### MICROBIAL INTERVENTION: WHAT IS IT?

It is the action or process of intervening biological processes in soil or in plants/plant roots by the micro organisms present in the rhizosphere which is mostly beneficial for enhancement of nutrient availability as well as growth and yield of crops.

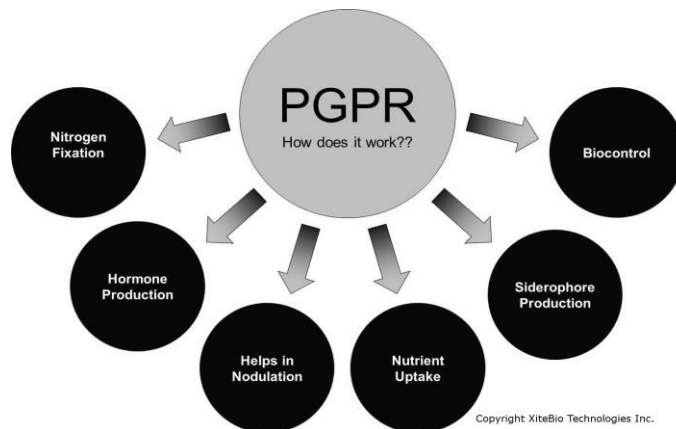
Microbial intervention may be helpful in attaining higher productivity with sustainability in agriculture in many ways, like: fixation of atmospheric nitrogen, increased availability of plant nutrients, decomposition and recycling of organic wastes and residues, bioaccumulation or microbial leaching of inorganics (Brierley 1985; Ehrlich 1990), suppression of soil-borne pathogens, bio-degradation of toxicants including pesticides, production of antibiotics and other bioactive compounds, production of simple organic molecules for plant uptake, complexation of heavy metals to limit plant uptake, solubilization of nutrient sources, production of polysaccharides to improve soil aggregation and many more.

This review article aims to cover the perspective of soil-beneficial bacteria and their role in plant growth promotion via direct and indirect mechanisms. Further elucidation of mechanisms involved will help to make these bacteria a valuable tool in agro-ecology in the near future.

### Plant growth promoting rhizobacteria

In the era of sustainable crop production, the plant-microbe interactions in the rhizosphere plays a pivotal role in transformation, mobilization, solubilization, etc. of nutrients from a limited nutrient pool, and subsequently uptake of essential nutrients by plants to realize their full genetic potential. At present, the use of biological approaches is becoming more popular as an additive to chemical fertilizers for improving crop yield in an integrated plant nutrient management system. In this regard, the use of plant growth promoting rhizobacteria (PGPR) has found a potential role in developing sustainable systems in crop production (Sturz et al., 2000; Shoebitz et al., 2009), though, the mechanisms of PGPR-mediated enhancement of plant growth and yield of many crops are not yet fully understood (Dey et al., 2004).

PGPRs have different relationships with different host plants. The two major classes of relationships are rhizospheric and endophytic. Rhizospheric relationships consist of the PGPRs that colonize the surface of the



**Figure 1.** Beneficial functions of PGPR. Source: <http://blog.xitebio.ca/6-ways-bacteria-promote-healthier-plants>.

root, or superficial intercellular spaces of the host plant, often forming root nodules. The dominant species found in the rhizosphere is a microbe from the genus *Azospirillum* (Bloemberg and Lugtenberg, 2001). Endophytic relationships involve the PGPRs residing and growing within the host plant in the apoplastic space (Vessy, 2003).

PGPR also help in solubilization of mineral phosphates and other nutrients, enhance resistance to stress, stabilize soil aggregates, and improve soil structure and organic matter content. PGPR retain more soil organic N, and other nutrients in the plant-soil system, thus they help in reducing the need for N and P fertilizer and enhance release of the nutrients. Beneficial effects of PGPR have been depicted in Figure 1.

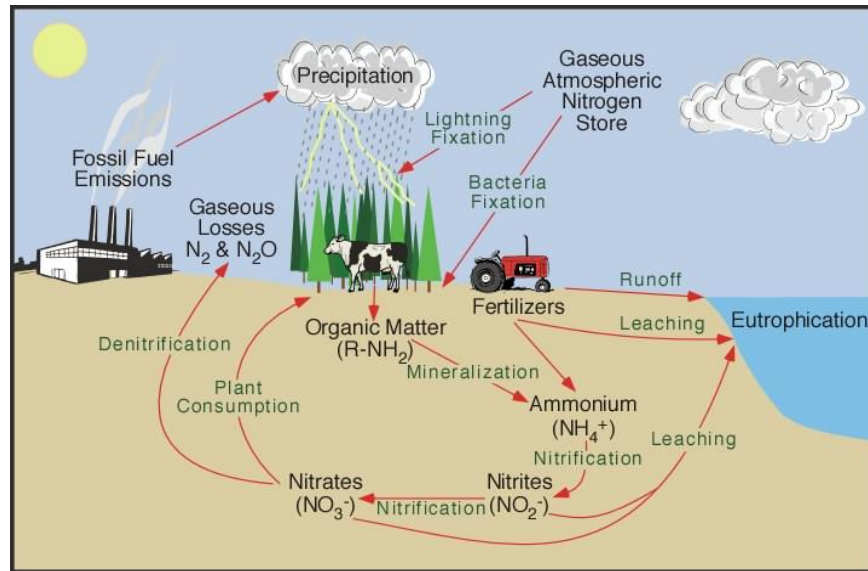
### Beneficial functions of PGPR

Direct plant growth promotion on the other hand, involves symbiotic and non-symbiotic PGPR functioning through production of plant hormones such as auxins, cytokinins, gibberellins, ethylene and abscisic acid. Production of indole-3-ethanol or indole-3-acetic acid (IAA), the compounds belonging to auxins, have been reported for several bacterial genera. Some PGPR function as a sink for 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene in higher plants, by hydrolyzing it into  $\alpha$ -ketobutyrate and ammonia, and in this way promote root growth by lowering indigenous ethylene levels in the micro-rhizo environment (Hayat et al., 2010).

#### Nutrient supply function

##### *Nitrogen fixing bacteria*

Nitrogen is one of the most important essential nutrient elements for plant growth and development but



**Figure 2.** Nitrogen Cycle in terrestrial ecosystems. Source: <http://www.physicalgeography.net>.

unfortunately is unavailable in its most prevalent form as atmospheric nitrogen. Plants instead depend upon combined or fixed forms of nitrogen, such as ammonia and nitrate. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide, ecological problems, such as the formation of coastal dead zones.

Biological nitrogen fixation, on the other hand, offers a natural means of providing nitrogen for plants (Wagner, 2012) (Figure 2).

### **Benefits of using biological nitrogen fixation (BNF)**

The process of biological nitrogen fixation (BNF) accounts for 65% of the nitrogen currently utilized in agriculture, and will continue to be important in future sustainable crop production systems (Matiru and Dakora, 2004). Important biochemical reactions of BNF occur mainly through symbiotic association of  $N_2$ -fixing microorganisms with legumes that converts atmospheric elemental nitrogen ( $N_2$ ) into ammonia ( $NH_3$ ) (Shiferaw et al., 2004). By inoculating legume seeds with appropriate rhizobia, farmers can ensure that they take advantage of the benefits of BNF listed below.

**1) Economics:** BNF reduces costs of production. Field trials have shown that the N captured by crops due to the use of rhizobia inoculants costing \$3.00/ha is equal to fertilizer N costing \$87.00.

**2) Environment:** The use of inoculants as alternatives to N fertilizer avoids problems of contamination of water

resources from leaching and run off of excess fertilizer. Utilizing BNF is part of responsible natural resource management.

**3) Efficiency:** Legume inoculants do not require high levels of energy for their production or distribution. Application on the seed is simple as compared to spreading fertilizer on the field. Long-term leguminous tree crops are self-sustaining through BNF.

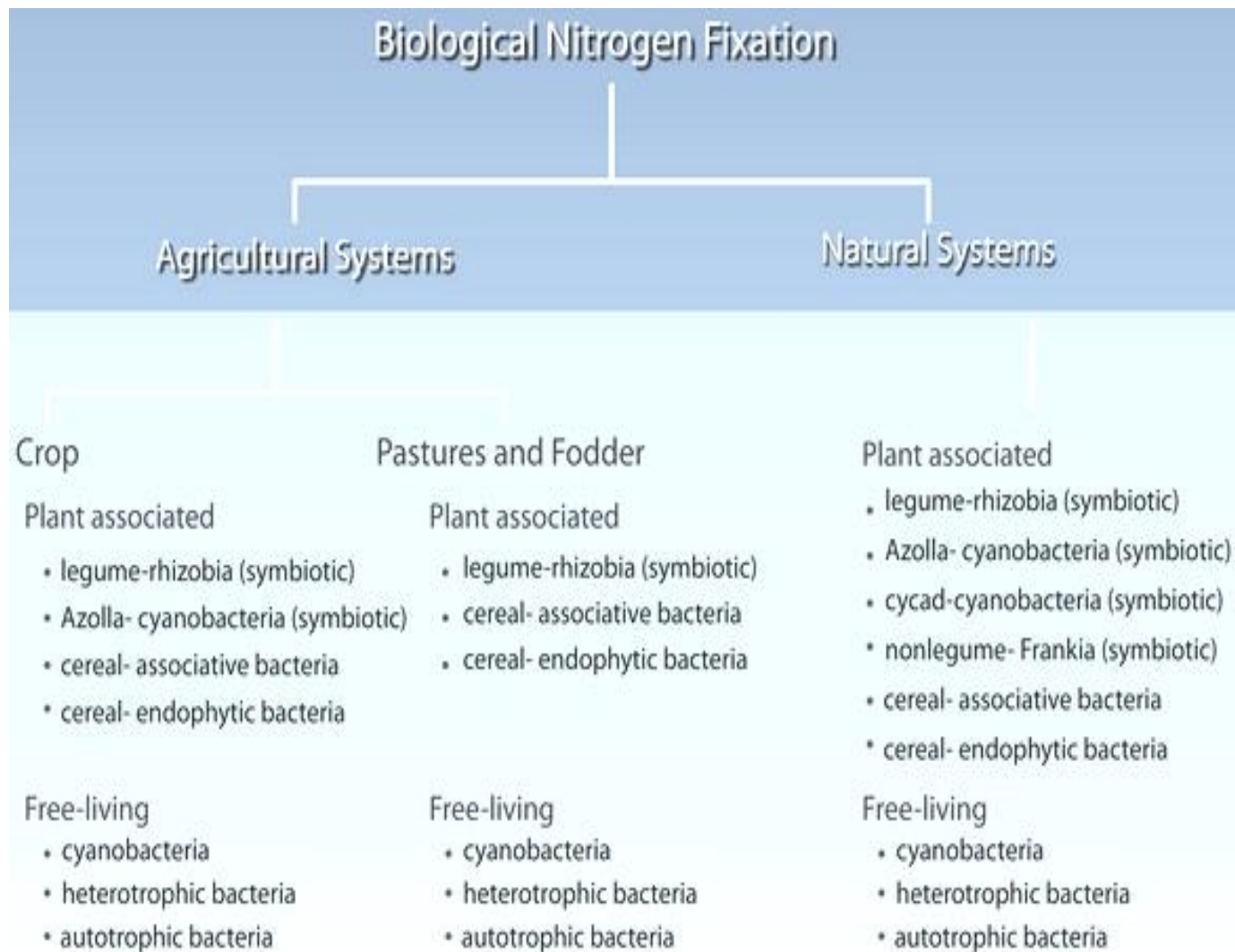
**4) Better yields:** Inoculants increase legume crop yields in many areas. BNF often improves the quality of dietary protein of legume seed even when yield increases are not detected.

**5) Increased soil fertility:** Through practices such as green manuring, crop rotations and alley cropping, N-fixing legumes can increase soil fertility, permeability, and organic matter to benefit non-legume crops.

**6) Sustainability:** Using BNF is part of the wise management of agricultural systems. The economic, environmental and agronomic advantages of BNF make it a cornerstone of sustainable agricultural systems. Legumes comprise one of the most important plant families in agriculture. Nitrogen-fixing members of this family include important food grains like soybeans, peas, beans and peanuts; forage crops like alfalfa and clover; and useful trees like leucaena and acacias (Silva and Uchida, 2000).

### **Types of micro-organisms involved in BNF at a glance**

Number of symbiotic as well as non-symbiotic (free living) micro-organisms, that are present in soil rhizosphere, can help in BNF in a number of crop and/or non-crop plants (Figure 3).



**Figure 3.** Types of microorganisms involved in BNF at a glance. Source: <http://www.nature.com/scitable/knowledge/library/biological-nitrogen-fixation-23570419>.

The nitrogen fixed by symbiotic *Rhizobia* in legumes can also benefit associated non-legumes via direct transfer of biologically fixed N to cereals growing in intercrops (Snapp et al., 1998) or to subsequent crops rotated with symbiotic legumes (Shah et al., 2003; Hayat, 2005; Hayat et al., 2008a, b). The plant nodule number and nodule weight increased with the age of the groundnut crop and highest was recorded at 60 days after sowing, when biofertilizer consortium was used with 10 t/ha of FYM (28.9 and 36.4 mg respectively) (Gunri and Nath, 2012). It was also found that biofertilizer application to red and lateritic soil of West Bengal, India, had a positive response to increase in pod and haulm yield of groundnut (Gunri et al., 2014). In many low input grassland systems, the grasses depend on the N<sub>2</sub> fixed by the legume counterparts for their N nutrition and protein synthesis, which is much needed for forage

quality in livestock production (Paynel et al., 2001; Hayat and Ali, 2010). In addition to N<sub>2</sub>-fixation in legumes, *Rhizobia* such as species of *Rhizobium* and *Bradyrhizobium* produce molecules (auxins, cytokinins, abscisic acids, lumichrome, riboflavin, lipochitooligosaccharides and vitamins) that promote plant growth (Hardarson, 1993; Herridge et al., 1993; Keating et al., 1998; Hayat and Ali, 2004; Hayat et al., 2008a, b). Their colonization and infection of roots would also be expected to increase plant development and grain yield (Kloepper and Beauchamp, 1992; Dakora, 2003; Matiru and Dakora, 2004). Other PGPR traits of *Rhizobia* and *Bradyrhizobia* include phytohormone production (Chabot et al., 1996a, b; Arshad and Frankenberger, 1998), siderophore release (Plessner et al., 1993; Jadhav et al., 1994), solubilization of inorganic phosphorus (Abd-Alla, 1994a; Chabot et al., 1996a) and



antagonism against plant pathogenic microorganisms (Ehteshamul-Haque and Ghaffar, 1993). Besides rice, *Rhizobia* have also been isolated as natural endophytes from roots of other non-legumes species such as cotton, sweet corn (McInroy and Kloepper, 1995), maize (Martinez-Romero et al., 2000), wheat (Biederbeck et al., 2000) and canola (Lupwayi et al., 2000) either grown in rotation with legumes or in a mixed cropping system involving symbiotic legumes.

A range of non-symbiotic plant growth promoting rhizobacteria (PGPR) participate in interaction with C<sub>3</sub> and C<sub>4</sub> plants (e.g., rice, wheat, maize, sugarcane and cotton), and significantly increase their vegetative growth and grain yield (Kennedy et al., 2004). *Azotobacter* species (*Azotobacter vinelandii* and *Azotobacter chroococcum*) are free-living heterotrophic diazotrophs that depend on an adequate supply of reduced C compounds such as sugars for their energy source (Kennedy and Tchan, 1992). Their activity in rice culture can be increased by straw application (Kanungo et al., 1997), presumably as a result of microbial breakdown of cellulose into cellobiose and glucose. Yield of rice (Yanni and El-Fattah, 1999), cotton (Iruthayaraj, 1981; Patil and Patil, 1984; Anjum et al., 2007), and wheat (Soliman et al., 1995; Hegazi et al., 1998; Barassi et al., 2000) increased with the application of *Azotobacter*.

In contrast to *Azotobacter*, *Clostridia* are obligatory anaerobic heterotrophs only capable of fixing N<sub>2</sub> in the complete absence of oxygen (Kennedy and Tchan, 1992; Kennedy et al., 2004). *Clostridia* can usually be isolated from rice soils (Elbadry et al., 1999), and their activity also increased after returning straw to fields, raising the C to N ratio in the soil.

*Azospirillum* species are aerobic heterotrophs that fix N<sub>2</sub> under microaerobic conditions (Roper and Ladha 1995) and grow extensively in the rhizosphere of gramineous plants (Kennedy and Tchan, 1992; Kennedy et al., 2004). Beneficial effects of inoculation with *Azospirillum* on wheat yields in both greenhouse and field conditions have been reported (Hegazi et al., 1998; El Mohandes, 1999; Ganguly et al., 1999). Inoculation with *Azospirillum brasilense* significantly increases cotton plant height and dry matter under greenhouse conditions (Bashan, 1998). Soil applications with *Azospirillum* can significantly increase cane yield in both plant and ratoon crops in the field (Shankariah and Hunsigi, 2001). The PGPR effects also increase N and P uptake in field trials (Galal et al., 2000; Panwar and Singh, 2000), presumably by stimulating greater plant root growth. Substantial increases in N uptake by wheat plants and grain were observed in greenhouse trials with inoculation of *A. brasilense* (Islam et al., 2002). <sup>15</sup>N tracer techniques showed that *A. brasilense* and *Azospirillum lipoferum* contributed 7-12% of wheat plant N by BNF (Malik et al., 2002).

The genus *Burkholderia* comprises 67 validly published species, with several of these including *Burkholderia*

*vietnamiensis*, *Burkholderia kururiensis*, *Burkholderia tuberum* and *Burkholderia phynatum* being capable of fixing N<sub>2</sub> (Estrada-delos Station et al., 2001; Vandamme et al., 2002). When *B. vietnamiensis* was used to inoculate rice in a field trial, it increased grain yields significantly up to 8 t ha<sup>-1</sup> (Tran Van et al., 2000). There is also evidence that these organisms can produce substances antagonistic to nematodes (Meyer et al., 2000).

*Herbaspirillum* is an endophyte which colonises sugarcane, rice, maize, sorghum and other cereals (James et al., 2000). It can fix 31-45% of total plant N in rice (30-day-old rice seedling) and N from the atmosphere (Baldani et al., 2000). The estimated N fixation by *Herbaspirillum* was 33-58 mg tube<sup>-1</sup> under aseptic conditions (Reis et al., 2000). *Herbaspirillum seropedicae* also acts as an endophytic diazotroph of wheat plants (Kennedy and Islam, 2001), colonizing wheat roots internally between the cells.

Several species of family *Enterobacteriaceae* include diazotrophs, particularly those isolated from the rhizosphere of rice. These enteric genera containing some examples of diazotrophs with PGP activity include *Klebsiella*, *Enterobacter*, *Citrobacter*, *Pseudomonas* and probably several others yet unidentified (Kennedy et al., 2004).

### Few research work tables validating the beneficial effects of nitrogen fixers in fixation of atmospheric nitrogen in soil

It is clear from Tables 1 and 2 nitrogen fixers are capable of fixation of atmospheric nitrogen symbiotically worldwide under varied edapho-climatic conditions in different host crops from the family leguminosae. Not only that, various non-symbiotic BNF are also there which have reported increase in yield (up to 50%) in cereals (rice) too.

### Phosphorus-solubilizing bacteria

When compared with the other major nutrients, phosphorus is by far the least mobile and available to plants in most soil conditions. Although phosphorus is abundant in soils in both organic and inorganic forms, it is frequently a major or even the prime limiting factor for plant growth. The bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species, nutritional status of soil and ambient soil conditions. When phosphatic fertilizers are applied to the soil, they often become insoluble (more than 70%) and are converted into complexes such as calcium phosphate, aluminum phosphate and iron phosphate in the soil (Mittal et al., 2008). Crop plants can therefore utilize only a fraction of applied phosphorus, which

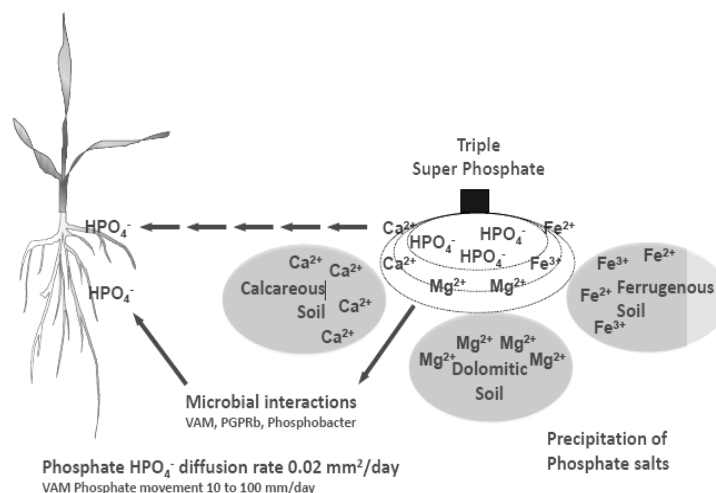
**Table 1.** A summary of biological nitrogen fixation measurements by different legumes.

Crop	Location	Crop N (Kg N ha <sup>-1</sup> )		N <sub>2</sub> fixation (%)
		Total N	N <sub>2</sub> Fixed	
Soybean	Brazil (Boddey et al., 1990)	112-206	85-154	70-80
	Hawaii (George et al., 1988)	120-295	117-237	80-97
Groundnut	Australia (Peoples and Craswell 1992)	171-248	37-131	22-53
	India (Giller et al., 1987)	126-165	109-152	86-92
Common bean	Brazil (Duque et al., 1995)	18-71	3-32	16-71
Cowpea	Indonesia (Sisworo et al., 1990)	25-69	9-51	12-33

**Table 2.** Increase in rice grain yield and estimated amounts of fixed N<sub>2</sub> by different N<sub>2</sub> fixing systems (Choudhury et al., 2004).

N <sub>2</sub> - fixator	Increase in rice yield		Estimated amount of N <sub>2</sub>
	Amount	(%)	
<i>Azolla-Anabaena</i> symbiosis	1.5 t ha <sup>-1</sup>	50	48.2 kg ha <sup>-1</sup>
<i>Cyanobacteria</i>	1.4 t ha <sup>-1</sup>	29	24.2 kg ha <sup>-1</sup>
<i>Azotobacter</i> sp	0.4-0.9 t ha <sup>-1</sup>	7- 20	11-15 kg ha <sup>-1</sup>
<i>Azospirillum lipoferum</i>	6.7 g plant <sup>-1</sup>	81	58.9% Ndfa
<i>Herbaspirillum</i> sp	3.7 - 7.5 g plant <sup>-1</sup>	45 - 90	38.1 – 58.2% Ndfa
<i>Burkholderia vietnamiensis</i>	0.6 - 7.9 g pot <sup>-1</sup>	13 - 22	Data not available
<i>Rhizobium leguminosarum</i>	0.6 - 7.9 g pot <sup>-1</sup>	2 - 22	23 – 31 mg

Ndfa: Nitrogen derived from the atmosphere.

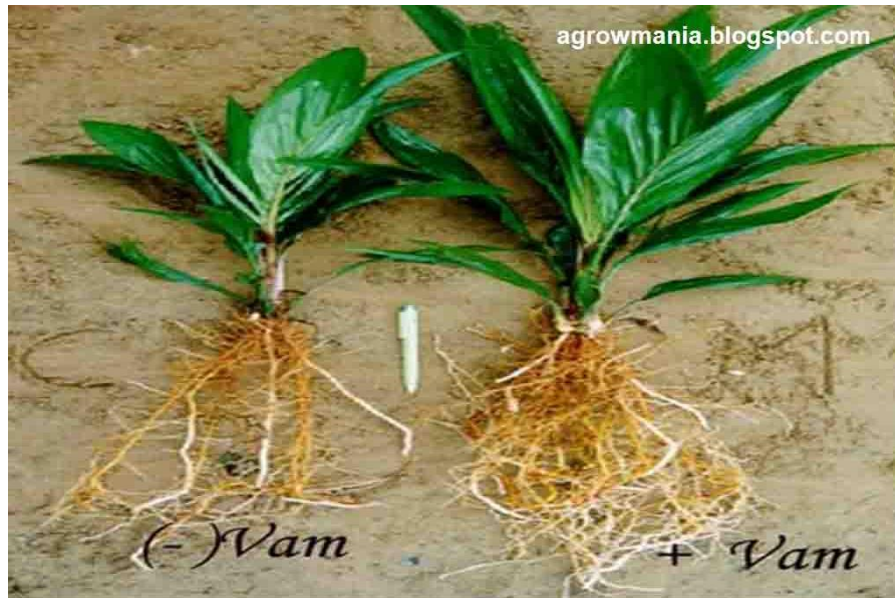
**Figure 4.** Microbes and phosphate conventional chemistry. Source: [http://otc.nfmf.no/public/news/12380\\_2.pdf](http://otc.nfmf.no/public/news/12380_2.pdf).

ultimately results in poor crop performance. To rectify this and to maintain soil fertility status, frequent application of chemical fertilizers is needed, though it is found to be a costly affair and also environmentally undesirable (Reddy et al., 2002).

To circumvent such phosphorus deficiency, phosphate-solubilizing microorganisms (PSM) could play an important role in supplying phosphate to plants in a more

environment-friendly and sustainable manner (Figure 4). It has been suggested that accumulated phosphates in agricultural soils is sufficient to sustain maximum crop yields worldwide for about 100 years (Walpola and Yoon, 2012). Therefore, using potential phosphate solubilizers can definitely be a solution to render this huge phosphate bank available to the plant community.

Bacterial strains belonging to genera the *Pseudomonas*,



**Figure 5.** Inoculation with VAM. Source: [http://agrowmania.blogspot.in/2009/06/biotech-solutions-to-organic\\_2227.html](http://agrowmania.blogspot.in/2009/06/biotech-solutions-to-organic_2227.html).

*Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium* and *Erwinia* have the ability to solubilize insoluble inorganic phosphate (mineral phosphate) compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite and rock phosphate (Goldstein, 1986; Rodríguez and Fraga, 1999; Rodríguez et al., 2006). Strains from genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers, while tricalcium phosphate and hydroxyl apatite seem to be more degradable substrates than rock phosphate (Arora and Gaur, 1979; Illmer and Schinner, 1992; Halder and Chakrabarty, 1993; Rodríguez and Fraga, 1999; Banerjee et al., 2006).

Integrated use of *Rhizobium*, PGPR containing ACC-deaminase in the presence of P-enriched compost would be a suitable approach for improving growth, yield and nodulation in lentil (Muhammad et al., 2012). Use of vesicular arbuscular mycorrhizae (VAM) is also getting importance in this context. These are special types of soil micorrizae that in association with plant roots, increase the root surface area and thereby improve soil-root contact, thus enhancing nutrient uptake by plants.

By applying VAM, the external mycelium extends several centimeters from the root surface and it then passes the depletion zone surrounding the root and exploits soil microhabitats beyond the nutrient depleted area where the small rootlets or root hairs cannot thrive. The phosphate is translocated into the mycelium in the root and is released for use by plants (Vishnu Sankar, 2009) (Figure 5).

Potassium (K) is the third major essential nutrient for plant growth. It plays an essential role for enzyme

activation, protein synthesis and photosynthesis. There are dynamic equilibrium and kinetic reactions between the different forms of soil K that affect the level of soil solution K at any particular time, and thus, the amount of readily available K for plants. Some microorganisms in the soil are able to solubilize 'unavailable' forms of K-bearing minerals, such as micas, illite and orthoclase, by excreting organic acids which either directly dissolve rock K or chelating silicon ions to bring the K into solution (Bennett et al., 1998; Barker et al., 1998). A wide range of rhizosphere bacteria namely *Pseudomonas*, *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *Bacillus edaphicus*, *B. circulans* and *Paenibacillus* sp. has been reported to release potassium in accessible form from potassium-bearing minerals in soils (Sheng, 2005; Lion et al., 2002; Li et al., 2006; Liu et al., 2012). These microorganisms are commonly known as potassium solubilizing bacteria (KSB) or potassium dissolving bacteria or silicate dissolving bacteria. Some research has been made on the use of potassium dissolving bacteria, known as "biological potassium biofertilizer (BPF)", particularly in China and South Korea to investigate the bio-activation of soil K-reserves so as to alleviate the shortage of K-fertilizer. It was shown that KSB increased K availability in soils and increased mineral uptake by plant (Sheng et al., 2002, 2003). Therefore, application of KSB holds a promising approach for increasing K availability in soils.

Inoculation with potassium solubilizing bacteria have been reported to exert beneficial effects on growth of cotton and rape (Sheng, 2005), pepper and cucumber (Han et al., 2006), sorghum (Badr et al., 2006), wheat (Sheng et al., 2006) and Sudan grass (Basak and and

wheat plants with *Bacillus mucilaginosus*, *Azotobacter chroococcum* and *Rhizobium* resulted in significant higher mobilization of potassium from waste mica, which in turn acted as a source of potassium for plant growth (Singh et al., 2010).

Chemical and spectroscopic studies have shown that in agricultural soils, most of the soil sulphur (>95%) is present as sulphate esters or as carbon-bonded sulphur (sulphonates or amino acid sulphur), rather than inorganic sulphate. Plant sulphur nutrition depends primarily on the uptake of inorganic sulphate. However, recent research has demonstrated that the sulphate ester and sulphonate-pools of soil sulphur are also plant-bioavailable, probably due to interconversion of carbon-bonded sulphur and sulphate ester sulphur to inorganic sulphate by soil microbes. In addition to this mineralization of bound forms of sulphur, soil microbes are also responsible for the rapid immobilization of sulphate, first to sulphate esters and subsequently to carbon-bound sulphur. The rate of sulphur cycling depends on the microbial community present, and on its metabolic activity, though it is not yet known if specific microbial species or genera control this process. The genes involved in the mobilization of sulphonate- and sulphate-ester sulphur by one common rhizosphere bacterium, *Pseudomonas putida*, have been investigated. Mutants of this species that are unable to transform sulphate esters show reduced survival in the soil, indicating that sulphate esters are important for bacterial S nutrition in this environment. *P. putida* S-313 mutants that cannot metabolize sulphonate-sulphur do not promote the growth of tomato plants as the wild-type strain does, suggesting that the ability to mobilize bound sulphur for plant nutrition is an important role of this species (Kertesz and Mirleau, 2004).

### Microbial intervention in soil-health improvement

Microorganisms, like different types of fungi, bacteria or actinomycetes present in soil help in degradation of soil organic matter and its ingredients like polysaccharides-cellulose, hemicelluloses lignin, pectin etc and finally lead to formation of amorphous colloidal materials which is known as humus.

Being highly colloidal and amorphous in nature, humus exhibit high CEC and WHC, also reduces bulk density and soil plasticity resulting in fluffy crumbly soil structure formation that is very much helpful for growing of crop plants.

### Microbial intervention in suppression of soil borne pathogens: Building microbial defense

Building and maintaining the diversity and activity of beneficial soil microbes produces a defensive network around the plant roots which out compete disease

organisms and provide protection for the plant. Some soil microorganisms can inhibit phyto-pathogens by the production of hydrocyanide (HCN) and/or fungal cell wall degrading enzymes, for example, chitinase and  $\beta$ -1,3-glucanase.

In addition, beneficial microbes can help suppress many root feeding pests during their juvenile growth stages by utilizing them as food resources. Further, in order to improve microbial defense in soil, few steps can be followed:

1. Soil and plant tonic containing a broad diversity of beneficial and predatory microbes, which is an effective way to build-up microbial numbers and diversity are used.
2. Biofoods and stimulants can also be added, which provides food and stimulation for beneficial soil microbes to build and strengthen the population once they are introduced.
3. Maintaining good levels of organic carbon will also provide a favourable habitat for beneficial microbes and encourage their proliferation and survival.

### Use of antibiotics

Many soil microorganisms develop antibiotics which help to destroy harmful pathogenic micro organisms and thereby support plant growth and development. For example, *Penicillium* sp., *Streptomyces* sp. present in soil produce penicillin and streptomycin, respectively, which inhibit the growth of many pathogenic micro organisms in soil by inhibition of cell wall, nucleic acid or protein synthesis, changes in metabolism, etc.

### Microbial intervention in detoxification function

This function can further be divided into:

- a) Complexation of heavy metals to limit plant uptake
- b) Degradation of toxicants in pesticides.

### Heavy metal detoxification

Heavy metal contamination due to natural and anthropogenic sources is a global environmental concern. Release of heavy metal without proper treatment poses a significant threat to public health because of its persistence, biomagnifications and accumulation in food chain. Non-bio degradability and sludge production are the two major constraints of metal treatment. Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and eco-friendly nature. Recent advances have been made in understanding metal- microbe interaction and their application for metal accumulation/detoxification

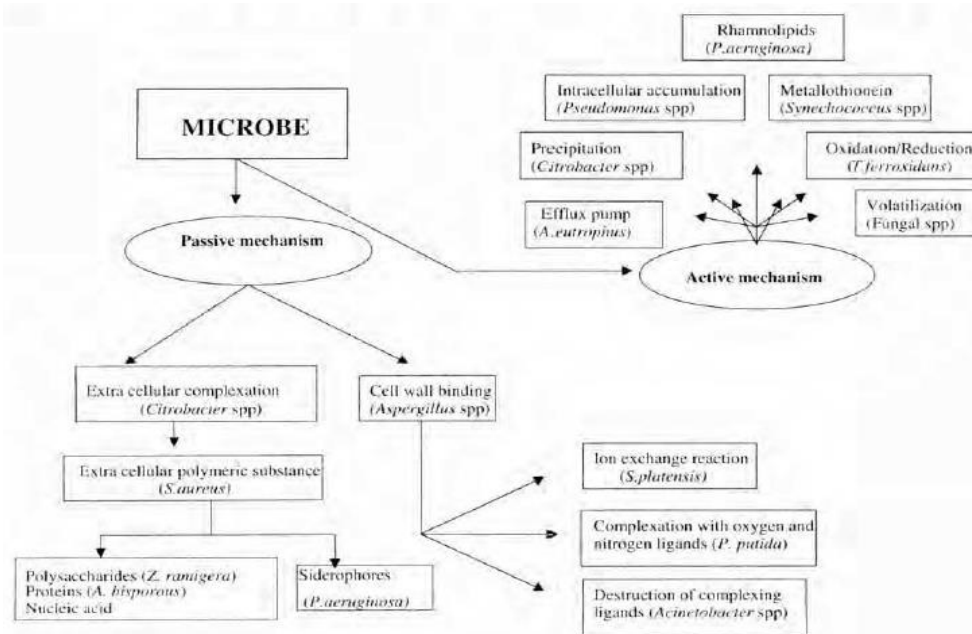


Figure 6. Mechanism of Microbial sorption.

(Rajendran et al., 2003).

There are a few metal elements (Ag, Cd, Sn, Au, Hg, Ti, Pb and Al) as well as metalloids (Ge, As, Sb and Se) that are considered as heavy metals and are found toxic in nature. The goal of microbial remediation of heavy metal contaminated soils and sediments are to immobilize the metal *in situ* to reduce metal bio-availability and mobility or to remove the metal from the soil. The mechanisms by which metal ions bind to the cell surface include electrostatic interactions, Van der Waals forces, covalent bonding, redox interactions and extracellular precipitation, or combination of these processes (Blanco, 2003).

Several active groups of cell constituents include acedamido group of chitin, structural polysaccharide of fungi (amino and peptidoglycosides), sulfhydryl and carboxyl groups in protein, phospho-diester (teichoic acid), phosphate, hydroxyl in polysaccharides, participate in biosorption (Vasudevan et al., 2001). Microbial mediated heavy metal sorption mechanisms are described in the Figure 6.

### Field applications of microbes in heavy metal toxicity bioremediation

The most important biotechnological application of metal-microbe interaction is in bioleaching, bioremediation, of polluted sites and mineralization of polluting organic matter.

Various microbially reducible metals, especially ferric iron in complexed form to keep it soluble at circum neutral pH, can be used as terminal electron acceptors in

*in situ* anaerobic bioremediation of sites polluted with toxic organics (Lovely, 1963). Fungi can convert oxidized selenium to volatile methylated selenides, to escape into the atmosphere (Frankenberger and Karlson, 1992), and bacteria can perform the methylation action on toxic arsenic metals resulting in their removal by volatilization. The increased rate of As (III) oxidation by native strains of *Bacillus* and *Geobacillus* might be exploited for the remediation of As in contaminated environments (Majumder et al., 2013). Twenty six arsenic (As) resistant bacterial strains were isolated from As contaminated paddy soil of West Bengal, India. Among them, 10 isolates exhibited higher arsenic resistance capacity and could be used as a potential bioremediator in future to combat with arsenic toxicity. Most probably these isolates were from *Bacillus* sp. (Majumder et al., 2013).

### Microbes in degradation of toxicants in pesticides

Soil microbes can also help in degradation and detoxification of harmful active ingredients of pesticides applied in various crops as well as activation of putatively pesticide organo-molecules. Heterotrophic microbes generally tend to derive energy from the carbon molecules of these compounds and thus trigger their activation or deactivations in general.

### CONCLUSIONS

Keeping in mind all these beneficial roles of microorganisms present in soil rhizosphere, it can be concluded

that in the integrated nutrient management (INM) system, integration of microbial inoculants with less fertilizer should be considered in many situations as it promises high crop productivity and agricultural sustainability. The commercial use of PGPR also must await the development of coating technology to improve methods of storing and applying bacteria without loss of viability. Novel, genetically-modified soil and region specific microbial intervention and technologies for their ultimate transfer to the fields have to be developed, pilot-tested and transferred to farmers in a relatively short time. And last but not the least, search for new strains of beneficial micro-organisms for bio-fertilizer and development of microbial diversity map for any region just like nutrient mapping may be helpful too. Advance simulation models related to nature of microbes and their behavioural patterns under changing edapho-climatic conditions may also be developed with suitable technical calibrations and testing for better development and maintenance of agricultural sustainability as well as microbial diversity in the near future.

### Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

## Endophytic mycobiota from leaves of *Indigofera suffruticosa* Miller (Fabaceae): The relationship between seasonal change in Atlantic Coastal Forest and tropical dry forest (Caatinga), Brazil

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Endophytic fungi were isolated from leaves of the medicinal plant, *Indigofera suffruticosa* collected at the Atlantic Coastal Forest and tropical dry forest (Caatinga), Pernambuco, Brazil, during the dry and rainy seasons. A total of 107 fungal isolates, representing nine fungal taxa, were obtained and classified as Ascomycota, among them *Colletotrichum gloeosporioides* with relative frequency (fr) 27.1% and *Pseudocochliobolus pallescens* with fr 16.82% were the most frequent. *Curvularia australiensis* and *Chaetomella raphigera* were isolated only in Caatinga during rainy and dry seasons, respectively, and for the first time they were isolated from a Caatinga plant. *Lasiodiplodia theobromae* was found only in Atlantic Coastal Forest in dry season, and according to Simpson (*D'*) and Shannon-Wiener (*H'*) indices fungal diversity were considered statistically significant in this forest. Besides, a greater similarity was observed between fungi isolated in Atlantic Coastal Forest and Caatinga in the dry season, suggesting the predominance of seasonality rather than geographical factor. This study is the first report on endophytes fungi from *I. suffruticosa*, and the results represent an important basis for further studies in the fields of ecology and biotechnology, since these endophytic fungi may be important source for future study in searching for new natural compounds with biological activities.

**Key words:** Ascomycota, Caatinga, endophytic mycobiota, fungal diversity, *Indigofera suffruticosa*, seasonal predominance.

### INTRODUCTION

Endophytic fungi are microorganisms that, during part or all of their life cycle, colonize inter and/or intracellularly healthy plant tissues, in an asymptomatic manner, without causing any apparent damage to their host (Tan and Zou, 2001), many of which are able to produce

secondary metabolites that may offer protection against different phytopathogens and herbivores (Rivera-Orduña et al., 2011). The endophytic fungi represent a wide diversity of microbial adaptations that have developed in special and unusual environments, making them a great

source of study and research for new chemicals for medicinal, industrial and agricultural uses (Aly et al., 2011; Kusari and Spiteller, 2011; Rajulu et al., 2011; Li et al., 2012; Kusari et al., 2013; Lou et al., 2013; Pharamat et al., 2013; Teiten et al., 2013). Furthermore, the production by the endophytic fungi of a variety of secondary compounds, such as alkaloids, terpenoids, steroids and aromatic compounds that are repellent or toxic to their enemies, gives greater competitive ability to colonized plants due to this symbiosis (Redman et al., 2002; Arnold et al., 2003; Rodriguez and Redman, 2008; Porrás-Alfaro and Bayman, 2011).

Clarke et al. (2006) demonstrated that plants colonized by endophytes have greater resistance as compared to non-colonized plants, and that endophytic fungi may be used to suppress plant diseases in various environments. Although this resistance mechanism is not fully understood, some studies have shown that climate variation and cultivation conditions influence vegetable metabolism (Simões-Ambrosio et al., 2010; Zalamea et al., 2013) and that seasonal variation and geographical location affect endophytic colonization (Martín et al., 2004; Göre and Bucak, 2007; Guo et al., 2008). Therefore, studies on endophytic fungi are of great importance for providing essential information for assessing fungal diversity under the influence of geographical and seasonal factors.

Few studies have been conducted with the communities of endophytic fungi in leaves of plants of the same species growing in different locations with distinct ecological characteristics (Collado et al., 1999; Martín et al., 2004; Göre and Bucak, 2007; Vaz et al., 2014). However, the composition of the endophytic mycobiota from different locations is very important for the understanding of the relationship between endophytic fungal populations and the establishment of plants subjected to distinct selection pressures in different ecosystems, mainly considering that endophytes may also increase the resistance of plants against biotic and abiotic stresses and produce compounds of biotechnological interest (Azevedo, 2014).

*Indigofera suffruticosa* Mill. is a shrub 1-2 m high originally from the Antilles and Central America, which was introduced and cultivated in Brazil on a large scale for the extraction of natural indigo dye for the textile industry, but in the 1980s this natural dye was replaced by an artificially produced pigment (Alzugaray and Alzugaray, 1988). *I. suffruticosa* is found in different biomes of Brazil, among these tropical dry forests (Caatinga) and Atlantic Coastal Forest. This plant is used in the folk medicine as a febrifuge, purgative, sedative (Almeida, 1993; Hastings, 1990) and to treat epilepsy, infection and inflammation such as gastrointestinal pain

(Roig, 1988; Matos, 1999; Agra et al., 2007). Previous studies have shown that the leaves of *I. suffruticosa* have embryotoxic effects (Leite et al., 2004), antimicrobial (Leite et al., 2006; Bezerra dos Santos et al., 2015) and anticonvulsant (Almeida et al., 2013) activities, and act as gastroprotective agent (Luiz-Ferreira et al., 2011).

Almost half of the world's tropical forests are represented by tropical dry forests. In Brazil, the tropical dry forests is named Caatinga due to the predominant type of vegetation (Albuquerque et al., 2012), and climate marked by high temperatures with sparse and irregularly distributed rains, with annual average precipitation ranging between 250 and 500 mm (Basso et al., 2005). The Caatinga soils are of different origins, and as a rule, are chemically fertile, well drained and oxygenated. Water bodies are rarely permanent, drying completely during the summer (Basso et al., 2005), but has faced intensive degradation from exhaustion due to deliberate introductions of exotic plants for giving support to farming activities (Leal et al., 2005). The rapid reduction of forests in tropical areas of Brazil is a major problem since this situation could result in the extinction of many endophytic fungi with the loss of potentially important products for use in agricultural, pharmaceutical, environmental and other fields of interest (Azevedo, 2014).

Another important biome of Brazil is the Atlantic Coastal Forest one of the most widely distributed tropical forests in Southern America, occupying almost all Brazilian Eastern coast besides inland areas and is marked by the occurrence of three important forest types (Oliveira-Filho and Fontes, 2000). That is characterized by a high biodiversity, and by proximity to the Atlantic Ocean, which provides a stable source of humidity, allowing high vegetation density. A floristic survey of the southern limit of Atlantic Forest area has been carried out, revealing several species of economic interest, many of which exhibit medicinal properties (Basso et al., 2005). Ribeiro et al. (2009) reported that due to the fragmentation process only 11.73% of the Atlantic Coastal Forest remains in Brazil, of which 12.1% is in Pernambuco State, where the sugar-cane plantations is among one of the main factors responsible for the fragmentation (Lôbo et al., 2011).

The isolation and identification of endophytic mycobiota is necessary, since the medicinal properties of a plant may be due to the ability of their endophytic microorganisms to produce biologically active secondary metabolites of medical and industrial interest, e.g. the taxol, which an anticancer agent produced by *Taxus brevifolia* Nutt., and by its endophyte fungus *Taxomyces andreanae* Strobel, A. Stierle, D. Stierle & W.M. Hess (Stierle et al., 1993; Bhardwaj and Agrawal, 2014).

In this context, the results of this research may

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contribute to future search on bioactive compounds derived from endophytic fungi species, since this is the first study on endophytic fungi colonization associated with *I. suffruticosa*. Thus, the objective of this study was to evaluate the relative geographical influence of two different locations and seasonality on the endophytic microbiota associated with *I. suffruticosa*.

## MATERIALS AND METHODS

### Plant material and study area

The collection of the plant material was done at natural growing in two areas studied in the rainy season (June) of 2009 and dry season (January) of 2010, at two different ecosystems. One collection site is located at the municipality of Caatinga (08°19'33"S, 36°04'21"W) in semiarid region of Pernambuco State, with average annual precipitation of 526.2 mm and a dry season that typically lasts nine months per year. The other site is in the municipality of Atlantic Coastal Forest (07°50'00"S, 34°54'30"W) in the coast of the Pernambuco State, with average annual precipitation of 1709.2 mm and a dry season that typically lasts three months per year (Rodal et al., 2008; APAC, 2013). Leaves of three health specimens of *I. suffruticosa* were randomly collected, in three different points of each areas studied and were put in plastic bags, transported to the laboratory, processed on up to 48 h for isolation and characterization of endophytic fungi according to methodologies established previously (Araújo et al., 2002). The plant material was authenticated by the Biologist Marlene Barbosa from the Botanic Department, Universidade Federal de Pernambuco (UFPE). A voucher specimen number 45217 has been deposited at the UFP Geraldo Mariz Herbarium of UFPE.

### Isolation of endophytic fungi

The plant material was subjected to a surface sterilization process in accordance with the methodology described by Araújo et al. (2002), where healthy leaves of *I. suffruticosa* were washed with running water, followed by immersion in 70% ethanol for one minute, sodium hypochlorite (2-2.5% active chlorine) for four minutes and washed three times in sterilized, distilled water. After surface sterilization, the samples were cut into fragments of 0.5 cm<sup>2</sup> and transferred aseptically to Petri dishes containing potato dextrose agar (PDA) culture medium supplemented with chloramphenicol (50 mg L<sup>-1</sup>) to suppress bacterial growth. The Petri dishes were inoculated each with six leaf fragments from different points of each area studied, in triplicate, were incubated at room temperature (28 ± 2°C) for 30 days, analyzed daily and any fungal colony present was isolated, purified and kept in PDA medium for subsequent identification. The control of efficiency of the sterilization method was confirmed by seeding 1 mL of the last washing in Petri dishes containing PDA medium.

### Identification of endophytic fungi

The morphological identification of endophytic fungi from *I. suffruticosa* was performed at the Micoteca URM, Department of Mycology, Federal University of Pernambuco, Recife, Brazil. The macro and micro morphological characteristics were observed based in technics and literature specific (Morton and Smith, 1963; Ellis, 1971, 1976; Sutton, 1980; Barnett and Hunter, 1987; Hanlin, 2000). After morphological identification, the representative fungi cultures were preserved in the Culture Collection – Micoteca URM

(WDCM604), Department of Mycology, Federal University of Pernambuco, Brazil.

### Data analysis

The frequencies of isolation of endophytic fungi were calculated. The absolute frequency (f) was estimated as the total number of endophytes isolates, and the relative frequency (fr) was the number of endophytes of each species divided by the total number of endophytic fungi. The rate of colonization was estimated as the total number of fragments of leaves colonized by fungi, divided for total number of fragments used for isolation of endophytes, as reported by Larran et al. (2002).

The number of isolates obtained was used to calculate the components of diversity: richness (S), number of different species found at each site and in each periods of the year, and evenness (*J'*), the Simpson (*D'*) and Shannon-Wiener (*H'*) diversity indices, as described by Magurran (1988) and the similarity matrix was constructed from the Sørensen index, which was grouped using UPGMA as clustering algorithm.

$$\text{Shannon-Wiener index } (H') = - \sum_{i=1}^s pi \ln pi$$

$$\text{Simpson index } (D') = 1 / \sum_{i=1}^s pi^2$$

$$\text{evenness } (J') = H' / \ln N$$

In the Shannon index, p is the proportion (n/N) of fungi of one particular species found (n) divided by the total number of fungi found (N), ln is the natural log, Σ is the sum of the calculations, and s is the number of species.

In the Simpson index, p is the proportion (n/N) of individuals of one fungal species found (n) divided by the total number of fungi found (N), Σ is still the sum of the calculations, and s is the number of species. The evenness was the ratio of observed diversity to maximum diversity (ln N), N is the total number of fungi found.

### Statistical analysis

Data were analyzed using Bioestat v. 5.0 by one-way analysis of variance (ANOVA) and Tukey test to determine statistical significance. A p-value of <0.05 was considered to be statistically significant.

## RESULTS AND DISCUSSION

From 216 fragments analyzed, of which 54 were collected at each site and in different periods of the year, a total of 107 fungal isolates, representing 9 fungal taxa, were obtained from isolations. The isolates were identified as Ascomycota and belong to the groups Pleosporales, Sordariomycetidae, Xylariales, Diaporthales, Leotiomyces and Bryosphaerales. Among them, the *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. was the taxon most frequent (27.41%), followed by *Pseudocochliobolus pallescens* Tsuda and Ueyama (16.82%), *Khuskia oryzae* H.J. Hunds (14.95%)

**Table 1.** Absolute (f) and relative (fr) frequency of endophytic fungi isolated from *I. suffruticosa* at Atlantic Coastal Forest and Caatinga (tropical dry forest) in rainy and dry season (Brazil).

Endophytic fungi	Atlantic Coastal Forest				Caatinga				Total	
	Rainy		Dry		Rainy		Dry		f	fr
	f	fr	f	fr	f	fr	f	fr		
<i>Colletotrichum gloeosporioides</i> (Penz.) Penz. & Sacc.			8	23.55	6	37.5	15	57.7	29	27.10
<i>Colletotrichum dematium</i> (Pers.) Grove (URM-6063)	4	12.9			2	12.5			6	5.60
<i>Pseudocochliobolus pallescens</i> Tsuda & Ueyama (URM-6064)	14	45.17			4	25.0			18	16.82
<i>Phomopsis archeri</i> B. Sutton (URM-5630)	2	6.45			2	12.5			4	3.74
<i>Curvularia australiensis</i> (Tsuda & Ueyama) Manamgoda, L. Cai & K.D. Hyde					2	12.5			2	1.87
<i>Pestalotiopsis maculans</i> (Corda) Nag Raj (URM-6061)	9	29.03	6	17.64					15	14.02
<i>Khuskia oryzae</i> H.J. Huds. (URM-6060)	2	6.45	6	17.64			8	30.77	16	14.95
<i>Chaetomella raphigera</i> Swift							3	11.53	3	2.80
<i>Lasiodiplodia theobromae</i> (Pat.) Griffon & Maubl.			3	8.82					3	2.80
Sterile mycelia			11	32.35					11	10.30
Total of endophytic fungi	31	100	34	100	16	100	26	100	107	100

and *Pestalotiopsis maculans* (Corda) Nag Raj (14.02%). The isolates that did not develop reproductive structures in medium culture were grouped as sterile mycelia (Table 1).

The species *P. pallescens*, *Phomopsis archeri* B. Sutton and *Colletotrichum dematium* (Pers.) Grove were detected in both sites and in particular only during the rainy season. Also, *Colletotrichum gloeosporioides* and *Khuskia oryzae* were detected in both sites, but not season association was observed. The species *P. maculans* presented locality specificity, since this species was isolated only in collections made in the Atlantic forest in both periods of the year (Table 1). Moreover, *Chaetomella raphigera* Swift, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Curvularia australiensis* (Tsuda & Ueyama) Manamgoda, L. Cai & K.D. Hyde exhibit specificity regarding the locality and period of the year. Specifically, *C. australiensis* and *C. raphigera* were isolated only in Caatinga during the rainy season and in the dry season, respectively, whilst *L. theobromae* was found only in Atlantic forest during the dry season (Table 1).

Incidental species are frequently observed in studies with endophytic fungi and represent those which have been isolated in a small number, as described by Siqueira et al. (2011). Incidental species were observed in the present study and included *C. australiensis*, *C. raphigera* and *L. theobromae*. The genus *Curvularia* was also found as incidental species in *Lippia sidoides* Cham. (Siqueira et al., 2011), and in the study of Bezerra et al. (2013) from the analysis of the endophytic fungi isolated from cactus *Cereus jamacaru* DC. growing in Caatinga,

Brazil. The species *L. theobromae* has been isolated as endophytes associated with *Piper hispidum* Sw. (Piperaceae) leaves (Orlandelli et al., 2012) and *C. raphigera* were also found as incidental species in *Catharanthus roseus* (L.) G. Don and *Cassia tora* L., herbaceous medicinal plants from the Malnad region, southern India (Krishnamurthy et al., 2008). Investigation of fungi isolated from plants that live in extreme environments is of paramount importance, since some studies have raised the hypothesis that endophytic fungi can alter the levels of the plant hormones that control stomatal behavior and osmotic adjustment (Malinowski and Belesky, 2000; Mandyam and Jumpponen, 2005). Given that plants are exposed to environmental stress due to factors such as low water availability, high salinity, high irradiance and nutrient deprivation, the association with these microorganisms can be the determining factor in the adaptation of plants to environmental conditions adverse.

In our study, the properties of the Atlantic Coastal Forest and Caatinga ecosystems present differences in several aspects. In terms of altitude, the municipality of Atlantic Coastal Forest is approximately 19 m a.s.l. (above sea level) and Caatinga approximately 552 m a.s.l. Comparing the type of soil, in Atlantic Coastal Forest, there is a predominance of floodplain type soil, while in Caatinga, clay types predominate. In terms of the vegetation around the site, the Caatinga is generally described as a woody vegetation with discontinuous canopy, variable in both height (3-9 m) and density, composed mostly of succulent (cacti essentially) and nonsucculent shrubs and trees, most of which are armed

with either thorns or prickles and bear microphyllous foliages, though they are leafless during the long-lasting periods of drought; the ground layer is rich in bromeliads annual herbs, and geophytes (Ab'Saber, 1974; Prado, 2003; Cardoso and Queiroz, 2011). According to data shown in Table 1, it can be seen that the environmental conditions found in Atlantic Coastal Forest may have favored more endophytic colonization than those found in Caatinga, since the number of fungi isolated in Atlantic Coastal Forest in the two seasons studied, was higher than the number of isolates from Caatinga in the same periods. Differences related to the number of species and specimens of endophytic fungi, in studies conducted in different locations and periods of the year, can be explained by the fact that the species involved can vary according to the host, geographic distribution, host plant age, ecological and seasonal conditions, including altitude and precipitation, and differences in the vegetation around the local where the plant was collected (Arnold et al., 2003; Helander et al., 2007).

Our results are in agreement with those observed in other studies, where it is reported that the population of endophytic fungi is directly related to the difference in intensity and period of exposure of these organisms to solar radiation, which generally occurs in areas that have differences in climatic factors (Collado et al., 1999; Martín et al., 2004; Martín Pinto et al., 2006; Hashizume et al., 2010). In addition, other environmental parameters such as soil, amount of available water, plant physiological condition at the time of collection and management, can also influence endophytic colonization (Hashizume et al., 2008).

The dominant endophytic fungi differed according to the collection sites and the period of the year (Table 1). In the municipality of Atlantic Coastal Forest, it was observed that the dominant endophytes differed according to the period of the year in which the collections were made. The fungus *C. gloeosporioides* was the dominant species in Caatinga in both periods of the year, unlike Atlantic Coastal Forest where *P. pallescens* was the dominant species in the rainy season and sterile mycelia was dominant in the dry season.

These results are in accordance with other studies of endophytes where it has been reported that many species of fungi can be isolated from a certain plant, but only a few are frequently found. This was the case, for example, in the study by Xing et al. (2010), which investigated the distribution and diversity of endophytic fungi in different tissues and ages of American ginseng. Among the 27 taxa isolated, *Glomerella* sp. was the dominant fungus in most tissues. In addition, Siqueira et al. (2011) analysing the endophytic mycobiota of leaves and stems of *L. sidoides* observed that *C. gloeosporioides* was the most frequent species in the leaves, while *Alternaria alternata* (Fr.) Keissl. was most frequent in stems. In a recent study, Vaz et al. (2014)

observed that *Pseudocercospora basintrucata* Crous and *Xylaria* sp. were the most frequent taxa isolated from the *Luma apiculata* (DC.) Burret in Andean Patagonian forest, while *Colletotrichum* sp. was the most frequent fungal species isolated from *Eugenia neomyrtifolia* M. Sobral in Atlantic rainforest, Brazil.

Studies in the semiarid region of the Brazilian northeast showed the endophytic associations of *Opuntia ficus-indica* (L.) Mill. and *Cereus jamacaru* with isolates of *K. oryzae* and species of the genus *Curvularia* (Bezerra et al., 2012, 2013). The genera *Curvularia* was also isolated by Oliveira et al. (2013) in a study on filamentous fungi diversity obtained from the soil of the semiarid area (Caatinga), Pernambuco, Brazil.

Although, the species *C. australiensis* and *C. raphigera* of endophytic fungi have been reported in isolates from other plant species, our study is the first to report on these fungi isolated from a plant of the semiarid region of Brazil. However, *C. australiensis* and *C. raphigera* were isolated as endophytic fungi in seed of *Withania somnifera* (L.) Dunal collected in a semiarid region of Pakistan (Khan et al., 2010). These species have also been isolated from leaves of *Ziziphus* sp. collected in the mountains of arid regions located at the South of the Arabian Gulf (El-Nagerabi et al., 2013). Nevertheless, in the semiarid region of Pernambuco, Brazil, the species *C. australiensis* was found in soil (Oliveira et al., 2013), and the genus *Drechslera* (nowadays known as *Curvularia*) was also isolated as endophyte in seeds of Cowpea (*Vigna unguiculata* (L.) Walp.), a plant of the Fabaceae family (Rodrigues and Menezes, 2002).

In our work, there was an occurrence of fungi that was not possible to sporulate in culture after a certain incubation period and they were classified as sterile mycelia. This was not a surprise since sterile mycelia were prevalent in several studies with endophytes (Xing et al., 2010; Siqueira et al., 2011; Sun et al., 2012; Bezerra et al., 2013). We hypothesized that the difficulty that some fungi have to develop reproductive structures is probably related to the fact that artificial culture media do not offer the same set of conditions that these fungi encounter in their host plants. These fungi have been identified in some studies with the aid of molecular biology (Guo et al., 2003; Giordano et al., 2009; Lacap et al., 2003), and the species classified as sterile mycelia in this study will also be identified through these tools in future studies.

Table 2 shows the diversity indices of endophytic fungal species isolated at different study sites and season, which compose three groups in accordance with the component of diversity that express richness (S), evenness (*J*) and dominance (D). The results obtained indicate that the richness of fungal species at the different study sites was S=5 during the rainy season, but showed variations in the dry season with S=4 in the Atlantic Coastal Forest and S = 3 in the Caatinga. The diversity was analyzed by Shannon-Wiener (*H'*) and Simpson (*D'*)

**Table 2.** Diversity, evenness and species richness of endophytic fungi isolated from *I. suffruticosa* at Atlantic Coastal Forest and Caatinga (tropical dry forest) in rainy and dry season (Brazil).

Site of collection	<i>H'</i>	<i>D'</i>	<i>J'</i>	S
<b>Atlantic coastal forest</b>				
Rainy season	1.334	3.215	0.388	5
Dry season	2.328	8.000	0.660	4
<b>Caatinga</b>				
Rainy season	1.490	4.048	0.537	5
Dry season	0.926	2.272	0.284	3

Shannon-Wiener (*H'*) and Simpson (*D'*) diversity indices, evenness (*J'*) and richness (S).

indices, and showed distinct variation of fungi, without repetition of the results at any of the study sites or period of the year. The highest diversity indices and evenness (*J'*) was found in Atlantic Coastal Forest in the dry season. Based on the Shannon-Wiener (*H'*) and Simpson (*D'*) indices, it is possible to see that there is a dominance of species during the rainy season as compared to the dry season in Caatinga was significant, while during the dry season in relation to the rainy season in Atlantic Coastal Forest, it was not significant. The differences in fungal endophytic assemblages observed in this study, indicate that variations in the study site and period of the year influence the species colonization and distribution in *I. suffruticosa* leaves, since the differences observed in the rainy and dry seasons in the different study sites were considered statistically significant. However, other factors may also influence endophytic colonization, such as the age of the plant collected, the differences in nutritional supply of host tissue and the differences in climatic conditions for example, relative humidity and intensity of light exposure (Talley et al., 2002; Vieira et al., 2011).

Moreover, some studies have indicated that the endophytic diversity in dry areas is low due to environmental factors, such as reduced rainfall and low vegetation density (Arnold et al., 2003; Suryanarayanan et al., 2002, 2003, 2005). For instance, Tejesvi et al. (2005) found only five species in a study with endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* (Roxb.) Wight & Arn. In a study on leaves and stems of plants from desert areas in China, Sun et al. (2012) found values for Shannon's index varying from 0.29 to 4.78, and for Simpson's index from 1.00 to 6.60. However, in contrast to our results, some studies have reported high endophytic diversity in plants of Caatinga, Pernambuco. A total of 71 fungi species involving 23 genera was found within four hundred seeds of Cowpea (*Vigna unguiculata* (L.) Walp) collected in Caruaru and Serra Talhada counties (Rodrigues and Menezes, 2002). Bezerra et al. (2012) studying the cactus *Opuntia ficus-indica* (L.) Mill. obtained 44 endophytic fungi, belonging to 12 genera and 13 species. In another

survey with cactus *C. jamacaru*, Bezerra et al. (2013) reported values for Shannon's index varying from 2.273 to 2.597, and for Simpson's index from 0.8127 to 0.9008.

According to the dendrogram shown in Figure 1, it is possible to perceive that there is a greater similarity between fungi isolated in Atlantic Coastal Forest and Caatinga in the dry season as compared to the rainy season, suggesting that it may be showing the predominance of seasonality rather than geographical factor.

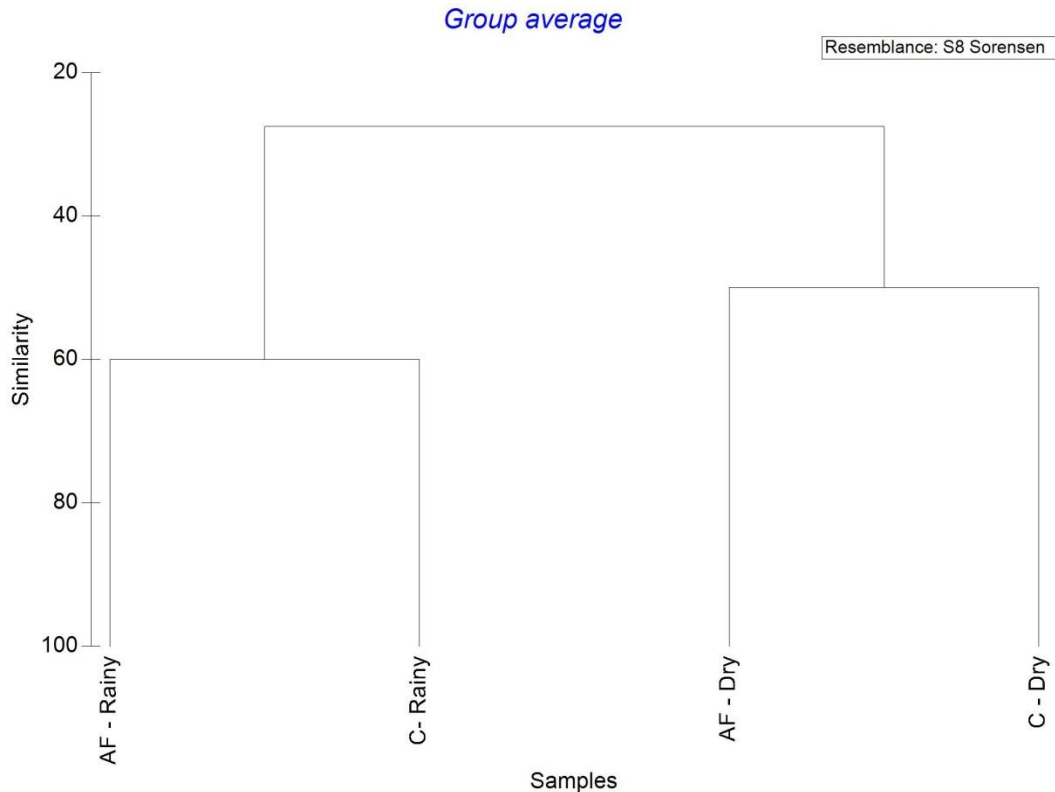
The results presented in this work are the first study of endophytic fungi from leaves of *I. suffruticosa* growing in Atlantic Coastal Forest and Caatinga in Brazil. This study documents that the properties of the Atlantic Coastal Forest biome associated with increased rainfall seem to favor greater endophytic colonization of *I. suffruticosa*, in comparison with the Caatinga. In this study, it is reported for the first time, the isolation of endophytic fungi *C. australiensis* and *C. raphigera* from a plant of the Brazilian Caatinga. Finally, the results indicate that there is a diversity of the endophytic fungi from *I. suffruticosa*, which are of ecological importance to plant growing in different areas studied, and also these fungi may be important source for future study in searching for new natural compounds with potential antimicrobial properties.

### Conflict of interests

The authors did not declare any conflict of interest.

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**Figure 1.** Dendrogram showing the relationship of communities of endophytic fungi isolated from *I. suffruticosa* at Atlantic Coastal Forest and tropical dry forest (Caatinga) in rainy and dry season (Brazil) from Sørensen similarity index. AF, Atlantic forest; C, Caatinga.

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Full Length Research Paper

# Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) modulate physiological mechanisms to tolerate drought stress due to arbuscular mycorrhizal fungi and mycorrhizal helper bacteria

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The influence of *Glomus intraradices* colonisation on growth and reactive oxygen metabolism of Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) seedlings treated with mycorrhizal helper bacteria, that is, phosphate solubilising bacteria containing a mixture of *Bacillus subtilis* and *B. megaterium*, *Azospirillum braisilence* or *Providencia* sp.) in potted culture was studied under well-watered or drought stress conditions. Results indicate that mycorrhizal inoculation increased plant growth and nutrient acquisition independent of the water regime, particularly when associated with mycorrhizal helper bacteria. Exposure of plant to drought stress led to generation of superoxide radicals and hydrogen peroxide in leaf tissues, however, their concentrations were lower in seedlings inoculated with *G. intraradices* and phosphate solubilising bacteria, as compared to other treatments including control. This particular treatment also increased total glutathione content and enhanced antioxidant enzyme activity in plant and microbial activity in soil. Mycorrhizal colonization was positively correlated with antioxidant metabolite, plant antioxidant enzymes, rhizospheric microbial activity and microbial biomass carbon and negatively correlated with reactive oxygen species under drought stress, which indicated that its inoculation could enhance plant defence system and alleviates oxidative damages to membrane lipids and proteins.

**Key words:** Cleopatra mandarin, superoxide radicals, hydrogen peroxide, anti-scavenging enzymes, plant nutrients, soil microbial biomass, soil microbial activity.

## INTRODUCTION

Drought stress is a major abiotic factor that limits plant's growth, thus becoming one of the growing concerns in

agriculture management around the world (Saeidnejad et al., 2013). The mycorrhizal association with roots of most

of the plants not only stimulate the plant growth, but also contribute in enhancing tolerance to drought (Navarro et al., 2011).

The free living and endo-symbiotic bacteria, known as “mycorrhizal helper bacteria” (MHB) stimulate pre-symbiotic fungal growth leading to an increase in root-fungus contacts and root colonization (Frey-Klett et al., 2007). The inoculation of *Glomus intraradices* along with PSB or *Azospirillum brasilense* can improve plant growth than single inoculation (Toro et al., 1997; Ruiz-Sanchez et al., 2011). *Providencia* sp., the bacterial strain AW5 (accession no FJ866760), used in the present investigation, was shown to have potential to stimulate plant growth through phosphorus solubilization, ammonia and indole-3-acetic acid production (Rana et al., 2011a).

Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) produces good quality fruits in scion variety for most of the citrus species (Ouko and Abubaker, 1988). The potential benefit of *G. intraradices* has been reported in Cleopatra mandarin (Camprubi et al., 1995). However, no work has been carried out to study the physiology of citrus plant under drought stress, when co-inoculated with AMF and MHB. Hence, *G. intraradices* alone or with MHB was used in the study so as to determine the suitable microbial combination for improving adaptive behaviour of Cleopatra mandarin against drought stress.

## MATERIALS AND METHODS

### Site of experimentation

The potted culture experiment was conducted in glasshouse at Indian Agricultural Research Institute (IARI), New Delhi, located at 77°12' E longitude, 28°40' N latitude and an altitude of 228.6 m above mean sea level. Climate is categorized as semi-arid, subtropical with hot dry summer and cold winter.

### Source of microbial inoculants

The AMF *G. intraradices* was procured from Division of Agricultural Microbiology, Kittur Rani Channamma College of Horticulture, Arabhavi, Karnataka. The starter culture was multiplied in ragi (*Elevusine coracana*), raised in plastic pot (12 × 20 cm) filled with a mixture of soil, sand and farm-yard manure (FYM) (2:2:1) that had been autoclaved (1.05 kg cm<sup>-2</sup>) for 2 h. The inoculum was sealed in a polythene packet consisting of freshly collected rhizosphere soil and AMF spores along with hyphae, arbuscules, vesicles and root segments of ragi plant.

*Providencia* sp. (AW5) was isolated from the wheat rhizosphere and identified using 16S rDNA sequence analysis in the division of Microbiology, IARI and submitted to NCBI (Accession No. FJ866760) (Rana et al., 2011b). Inoculum of bacterial strain was

prepared by growing in nutrient broth at 28 ± 1°C for 48 h at 100 rpm, such that the inoculum contained 10<sup>11</sup> cells ml<sup>-1</sup>.

The other bacteria like *A. brasilense* and PSB (*Bacillus subtilis* + *Bacillus megaterium*) were cultured in nutrient broth and then multiplied in carrier like finely powdered and sterilized charcoal powder. The broth containing 10<sup>9</sup> cells ml<sup>-1</sup> was added to 1/3 of the water holding capacity of the carrier.

### Plant material, microbial inoculation and growth conditions

The seeds of Cleopatra mandarin were collected from the germplasm of Division of Fruits and Horticultural Technology, IARI and then surface sterilized by immersing in 70% alcohol for 5 min, followed by rinsing three times with sterile distilled water and then kept over wet filter paper in Petri dishes at 28°C for germination. After 7 days, the seedlings were planted in plastic containers (12 × 20 cm) containing 4.1 kg of mixture of sterilised soil : sand : FYM (2:2:1) having EC<sub>(1:2)</sub> 6.35 mS m<sup>-1</sup>, pH<sub>(1:2)</sub> 7.92, HCO<sub>3</sub><sup>-</sup> 1.14 g kg<sup>-1</sup> and Cl<sup>-</sup> 5910.75 ppm.

During planting, the seedlings were inoculated with either AMF (5 g per kg of potting mixture consisting of 80 to 88 infective propagules per 5 g of inoculum) or bacterial species (5 g per kg of potting mixture containing 10<sup>9</sup> cells per g of carrier) or both. In case of *Providencia* sp. (AW 5), 5 ml of liquid media containing 10<sup>11</sup> cells per ml of media was used for each pot. The seedlings were maintained in glasshouse with day-night temperatures of 27 ± 1°C and humidity of 80-85%.

Day lengths were extended up to 16 h with cool white fluorescent lights at 630 μmol m<sup>-2</sup> s<sup>-1</sup> for improving the vegetative growth of the plants. Seedlings were watered on alternate days with 250 ml of autoclaved water, maintaining soil moisture content above 80% using soil moisture meter. Water used for irrigation in high density orchard had EC<sub>(1:2)</sub> 288 μS m<sup>-1</sup>, pH<sub>(1:2)</sub> 7.48, HCO<sub>3</sub><sup>-</sup> 1.0 milliequivalent/litre and Cl<sup>-</sup> 110.76 ppm.

### Imposition of differential moisture regimes

The differential water treatments were started at 270 DAI under glasshouse condition. Half of seedlings under each treatment received ample water (750 ml) at an interval of two days and the remaining half were imposed drought stress by withholding water. Daily soil relative water content was measured using soil moisture meter (FieldScout TDR 300, Spectrum Technologies, Inc.) fitted with 4.8-inch probe rods. Wet point was fixed at 90% and dry point at 8%. The soil relative water content (RWC) for well-watered (WW) seedlings was monitored at 80%. Entire plants were harvested after 20 days, when drought stressed (WS) seedlings showed visible symptoms of temporary wilting.

### Measurements

#### Root colonization

Per cent root colonization was determined by using the method as detailed by Phillips and Haymann (1970). The presence of fungal hyphae, arbuscules and vesicles were observed under 10x

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**Abbreviations:** AMF, Arbuscular mycorrhizal fungi; MHB, mycorrhizal helper bacteria; PSB, phosphate solubilising bacteria; WS, water stress; WW, well-watered; DAI, days after inoculation.

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microscope (Nikon TS 100).

### Fresh and dry weight of shoot and root

Fresh weight of root and shoot of each plant was recorded by electronic balance. The samples were then put in the perforated paper bag and kept in hot air oven at 70°C until constant dry weight.

### Leaf nutrient analysis

For tissue nutrient analyses, oven-dried samples were ground, sieved and digested in nitric acid : perchloric acid (9:4). Total nitrogen (N) was determined in samples of 0.5 g dry weight using the Kjeldahl method. Phosphorous (P) was analysed by a vanadatemolybdate method. The P transmittance was read at 420 nm. Potassium (K) was determined with the help of flame photometer (Systronics 128, Ahmedabad) using specific filter and LPG flame. Determination of other foliar nutrients like Ca, Mg, Zn, Fe, Cu and Mn was done by the atomic absorption spectrophotometer (GBC-Avanta PM; GBC-Advanta Scientific Equipment, Dandenong, Victoria, Australia) using nitrous oxide-acetylene flame.

### Reactive oxygen species

Leaf samples (1 g) were homogenised in 5 ml of pre-cooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM diethyl dithiocarbamate and centrifuged at 5000 g for 5 min. Superoxide radical was estimated as per the method of Chaitanya and Naithani (1994). Hydrogen peroxide was estimated as per the method of Rao et al. (1996).

### Antioxidant enzymes

Aliquots (1 g) of roots were homogenized in 5 ml of chilled phosphate buffer (0.1 mol l<sup>-1</sup>, pH 7.8) and centrifuged at 15,000 g for 20 min at 4°C (Hermle Z 323K), and the supernatant was used for enzyme assays. The activity of catalase (CAT, EC 1.11.1.6) was estimated by titrating the reaction mixture containing 1 ml of enzyme extract and 2 ml of H<sub>2</sub>O<sub>2</sub> against KMnO<sub>4</sub> (0.01 M) till the appearance of faint pink colour persisting at least for 15 s (Luck, 1974). The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured as a decrease in absorbance at 290 nm as ascorbate was oxidised by H<sub>2</sub>O<sub>2</sub> (Nakano and Asada, 1981). Guaiacol-peroxidase (G-POD, EC 1.11.1.7) activity was measured by following the change of absorption at 560 nm due to guaiacol oxidation (Thomas et al., 1981). The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Beyer and Fridovich (1987). Glutathione reductase (GR, EC 1.8.1.7) assay was determined based on the formation of reduced glutathione with 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), which absorbs at 412 nm, as proposed by Carlberg and Mannervik (1985). Protein concentrations were determined by a modified Lowry method (Hartree, 1972) with bovine serum albumin as a standard protein.

### Non-enzymatic antioxidant

Total glutathione (GSH + GSSG), an important antioxidant, was determined according to the method of Griffith (1985) and Smith (1985).

### Soil microbiological parameters

Alkaline phosphatase activity was assayed as per the method of Tabatabai and Bremner (1969) and the enzymatic activity was expressed as µg of p-nitrophenol g<sup>-1</sup> soil dry weight h<sup>-1</sup>. Dehydrogenase activity, expressed as µg of triphenyl formazon g<sup>-1</sup> soil dry weight day<sup>-1</sup>, was assayed as per the method of Casida et al. (1964). Microbial biomass carbon (MBC), expressed as µg g<sup>-1</sup> soil sample, was estimated by the method of Nunan et al. (1998).

### Experimental design and statistical analysis

Experiments were laid out in a completely randomised block design with eight treatments and three replications per treatment. The treatment details are as follows: T<sub>1</sub> = Control; T<sub>2</sub> = PSB alone; T<sub>3</sub> = *A. brasilense* alone; T<sub>4</sub> = *Providencia* sp. (AW5) alone; T<sub>5</sub> = *G. intraradices* alone; T<sub>6</sub> = *G. intraradices* and PSB; T<sub>7</sub> = *G. intraradices* and *A. brasilense*; T<sub>8</sub> = *G. intraradices* and *Providencia* sp. (AW 5).

Experimental data from a period of two years was pooled and then subjected to analysis of variance (ANOVA) using statistical analysis software SPSS package (SPSS 11.0) and means were evaluated by Fisher's protected least significant difference (LSD). Differences at P < 0.05 were considered significant.

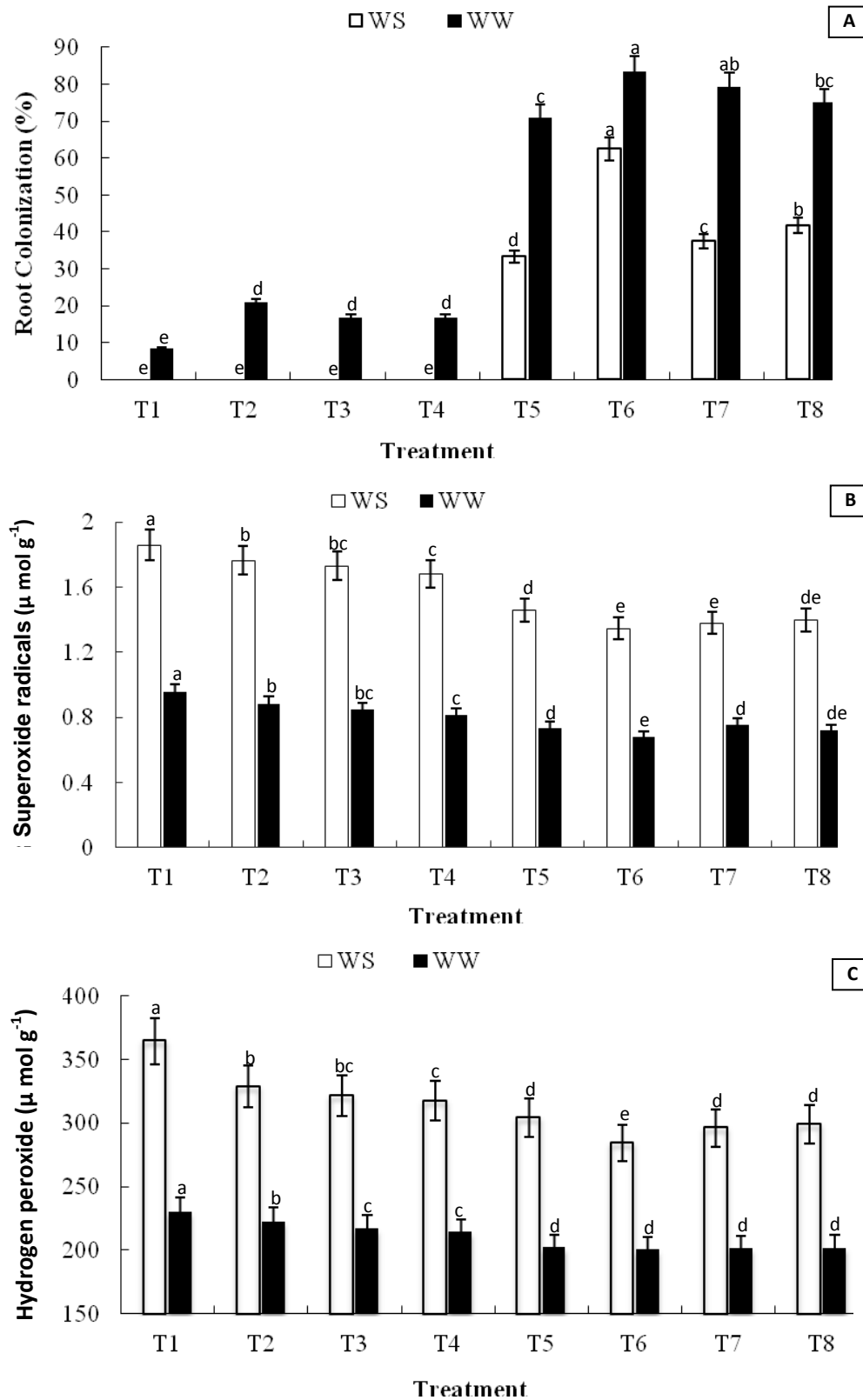
## RESULTS

Drought stress had a strong effect on AMF development. The present study revealed significantly highest root colonisation in seedlings co-inoculated with *G. intraradices* and PSB under WS and WW conditions (62.50 and 83.33%, respectively). Colonization was not observed in the roots of non-AM seedlings under WS (Figure 1A).

Shoot and root weight was reduced under drought stress, irrespective of the treatment provided (Table 1). However, co-inoculation of *G. intraradices* and PSB increased respectively fresh and dry weight of shoot (281.60 and 377.94%) and that of root (547.98 and 578.18%), as compared to control. Under WW condition, fresh and dry weight of shoot was increased respectively by 221.57 and 213.66% and that of root by 309.34 and 367.35%, respectively, in seedlings co-inoculated with *G. intraradices* and PSB.

The super oxide radical (O<sub>2</sub><sup>-</sup>) in leaf was found to be produced more under WS condition, as compared to WW condition, regardless of any treatment (Figure 1B). However, production was significantly lesser by 27.69 and 28.80% in seedlings co-inoculated with *G. intraradices* and PSB, as compared to control, under WS and WW conditions, respectively, which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense*, *G. intraradices* and *Providencia* sp. (AW 5) under WS condition (25.81 and 24.73%, respectively) and *G. intraradices* and *Providencia* sp. (AW 5) under WW condition (24.61%).

The accumulation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was reduced by 21.97% in seedlings co-inoculated with *G. intraradices* and PSB, as compared to control (Figure



**Figure 1.** Influence of microbial inoculants on (A) AMF colonization in root (12 root segments observed per treatment); (B) Superoxide radicals; (C) hydrogen peroxide content in fresh leaves of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at  $p \leq 0.05$ ,  $n = 2$  (Web Agri Stat Package 2.0).

**Table 1.** Response of Cleopatra mandarin to microbial inoculants on shoot and root weight (g plant<sup>-1</sup>).

T	Fresh shoot weight		Fresh root weight		Dry shoot weight		Dry root weight	
	WS	WW	WS	WW	WS	WW	WS	WW
T <sub>1</sub>	5.11±0.84f	11.15±0.32f	2.59±0.57e	7.55±0.08f	2.68±0.37f	6.26±0.32e	1.54±0.30f	3.40±0.22e
T <sub>2</sub>	8.84±0.50e	13.96±0.57e	5.90±0.86d	7.56±0.50f	4.77±0.50e	6.91±0.57de	1.93±0.42ef	3.68±0.21e
T <sub>3</sub>	11.70±0.06d	14.34±0.52e	9.62±0.11c	10.52±0.01e	6.69±0.06d	8.79±0.23c	3.31±0.39d	6.06±0.04cd
T <sub>4</sub>	11.09±0.10d	13.63 ±0.57e	8.45±0.24c	9.54±0.23e	5.46±0.10e	7.88±0.28cd	2.85±0.05de	5.09±0.03d
T <sub>5</sub>	12.11±0.26d	20.32± 0.75d	10.10±0.06c	11.82±0.04d	7.29±0.03d	12.74±0.32b	4.67±0.15c	7.10±0.25c
T <sub>6</sub>	19.51±0.32a	35.86±0.81a	16.79±0.51a	30.89±0.47a	12.83±0.32a	19.64±0.52a	10.41±0.51a	15.89±0.53a
T <sub>7</sub>	16.65±0.64b	26.82±0.83b	13.34±0.75b	19.82±0.49b	10.61±0.64b	13.50±0.81b	6.63±0.23b	11.18±0.72b
T <sub>8</sub>	14.28±0.18c	23.38±0.81c	12.42±0.87b	16.74±0.44c	8.59±0.12c	13.46±0.29b	5.94±0.06b	10.36±0.72b

Mean ± standard error, n = 2; values followed by same letter in a column are not significantly different (p ≤ 0.05).

1C). Under WW condition, H<sub>2</sub>O<sub>2</sub> content was significantly lesser in seedlings co-inoculated with *G. intraradices* and PSB, *G. intraradices* and *A. brasilense*, *G. intraradices* and *Providencia* sp. (AW 5) and *G. intraradices* alone (12.83, 12.30, 12.21 and 12.08%, respectively), as compared to the control.

Drought stress had positive effect on increase in activities of leaf enzymes in Cleopatra mandarin (Figure 2A, B, C, 3A and 3B). The co-inoculation of *G. intraradices* and PSB enhanced the activities of CAT (53.60%), APX (46.85%), G-POD (27.77%) and GR (73.25%), which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense* for CAT and G-POD (51.36 and 26.39, respectively) and *G. intraradices* and *A. brasilense*, *G. intraradices* and *Providencia* sp. (AW 5) and *G. intraradices* alone for APX (42.31, 41.43 and 38.13, respectively), whereas *G. intraradices* in combination with *A. brasilense* or PSB exhibited increased SOD activity by 74.01 and 71.60%, respectively, as compared to control under WS condition. Under WW condition, activities of CAT, APX, G-POD, SOD and GR were respectively increased by 10.58, 17.55, 22.64, 141.70 and 114.89% in seedlings co-inoculated with *G. intraradices* and PSB, as compared to control, which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense* and single inoculation of *G. intraradices* for CAT (9.19 and 9.05%, respectively) and co-inoculation of *G. intraradices* and *A. brasilense* for APX (16.64%).

The co-inoculation of *G. intraradices* and PSB significantly stimulated highest increase in total glutathione in leaf under WS and WW conditions (24.47 and 27.82%, respectively), as compared to control, which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense* (22.24%) under WS (Figure 3C).

The leaf nutrient content was found to be influenced by microbial inoculation, regardless of any moisture regime. The leaf N content was 58.19 and 38.13% higher in seedlings co-inoculated with *G. intraradices* and *A. brasilense* under WS and WW, respectively, as

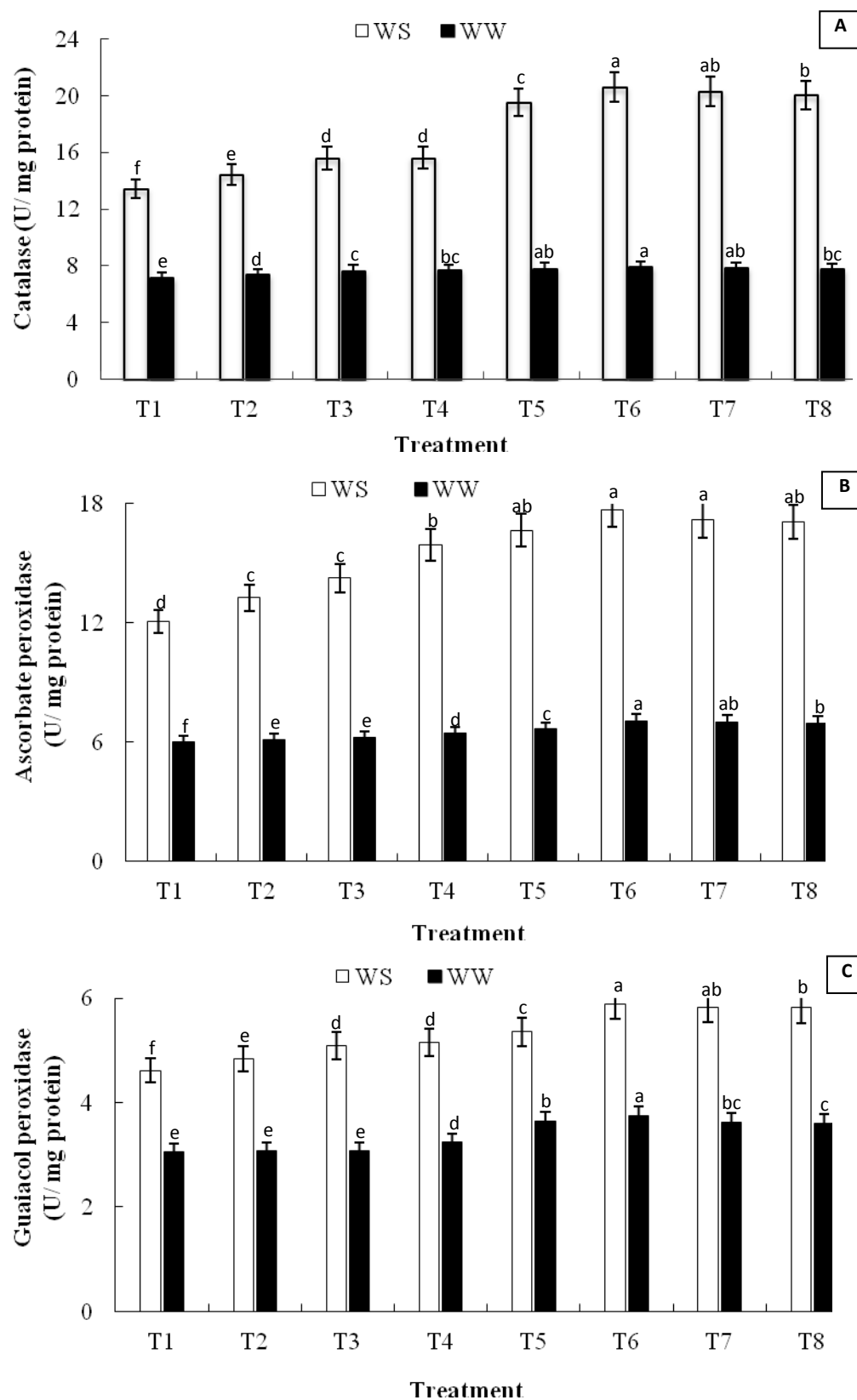
compared to the control, which was statistically at par with seedlings inoculated with *Azospirillum* alone (34.92%) under WS (Table 2). The synergistic effect of *G. intraradices* and PSB resulted in significant increase in leaf P, K, Ca and Mg content, regardless of any moisture regime, as compared to the control, which was statistically at par with inoculation of *G. intraradices* alone and *Azospirillum brasilense* alone under WS and WW, respectively, for Ca content, inoculation of *G. intraradices* alone and co-inoculation of *G. intraradices* and *A. brasilense* under WW condition for Mg content (Table 2).

The drought stress also reduced leaf micronutrient content. However, inoculation of *G. intraradices* along with PSB resulted in significantly higher level of leaf Fe, Cu, Mn and Zn content, regardless of any moisture regime, which was statistically at par with co-inoculation of *G. intraradices* and *Providencia* sp. (AW 5) under WS for Cu content, co-inoculation of *G. intraradices* and *A. brasilense* under WS condition for both Mn and Zn content (Table 3).

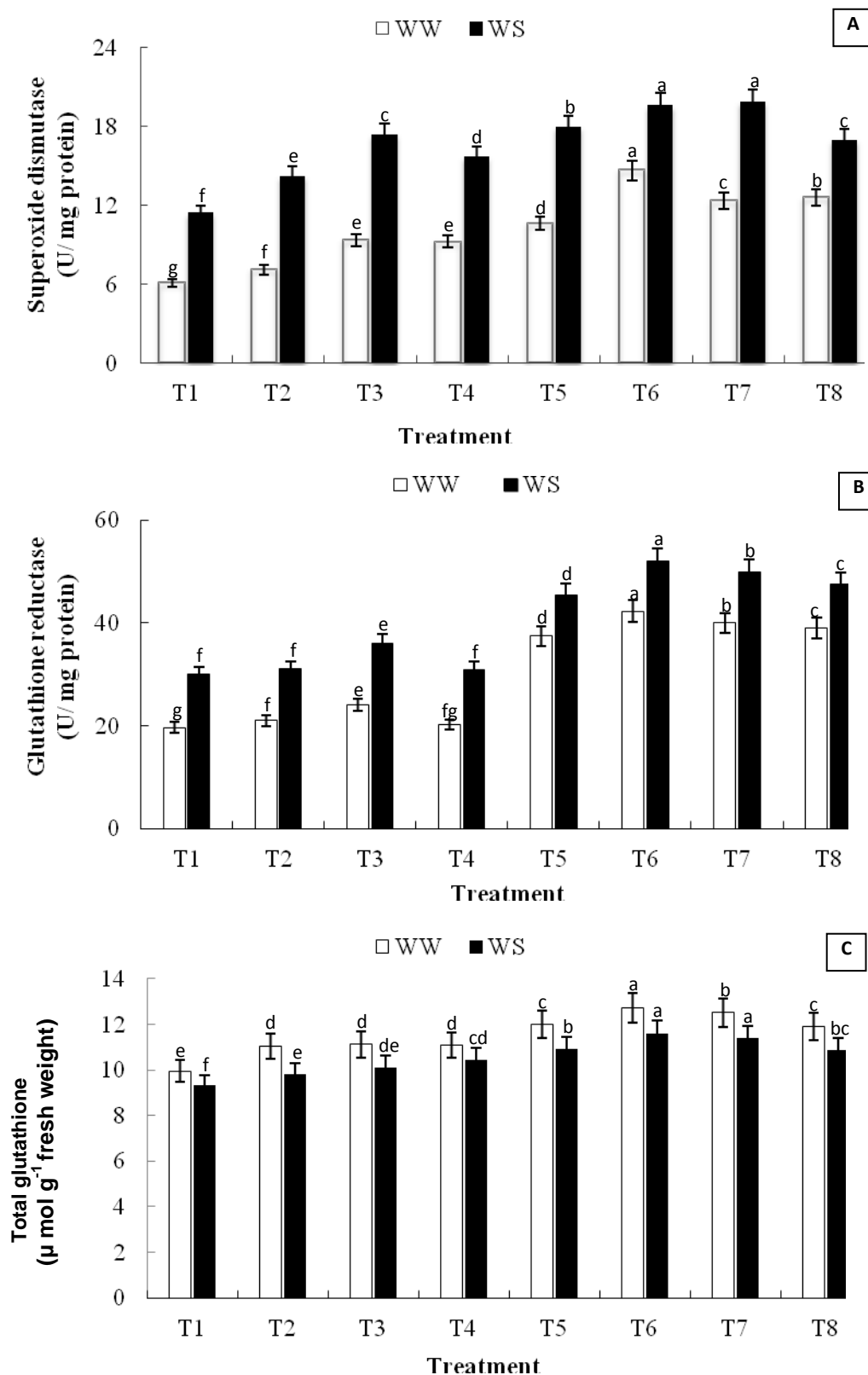
The analysis of rhizospheric soil inoculated with different microbial culture including control revealed the superiority of co-inoculation of AMF and MHB over single inoculation and in particular, co-inoculation of *G. intraradices* and PSB for alkaline phosphatase activity under WS and WW conditions (81.02 and 87.30%, respectively), as compared to the control (Figure 4A).

Drought stress had negative effect on soil dehydrogenase activity (Figure 4B). However, it had significantly lesser effect in the rhizosphere of seedlings inoculated with *G. intraradices* and PSB (61.55%) as compared to the control. The particular treatment also showed 49.61% more enzyme activity, as compared to the control, under WW condition.

Microbial biomass carbon (MBC) was affected by different microbial treatment, however, inoculation of AMF and MHB had significant effect than single inoculation or uninoculated control (Figure 4C). Under WS and WW conditions, the MBC content was 192.73 and 100.04% more in *G. intraradices* + PSB inoculated rhizosphere as compared to the control, which was



**Figure 2.** Influence of microbial inoculants on (A) Catalase; (B) Ascorbate peroxidase; (C) Guaiacol peroxidase content in fresh leaves of *Cleopatra mandarin* grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at  $p \leq 0.05$ ,  $n = 2$  (Web Agri Stat Package 2.0).



**Figure 3.** Influence of microbial inoculants on (A) superoxide dismutase; (B) glutathione reductase; (C) total glutathione content in fresh leaves of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at  $p \leq 0.05$ ,  $n = 2$  (Web Agri Stat Package 2.0).



**Table 2.** Response of Cleopatra mandarin to microbial inoculants on leaf macro nutrient content (%).

	N		P		K		Ca		Mg	
	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW
T <sub>1</sub>	1.16±0.03f	1.48±0.08d	0.18±0.01e	0.25±0.02e	1.40±0.01f	1.80±0.01g	1.48±0.03e	1.28±0.03f	0.10±0.01d	0.18±0.02c
T <sub>2</sub>	1.36±0.04e	1.70±0.01c	0.24±0.02d	0.27±0.01de	1.65±0.02d	2.04±0.01d	1.61±0.05d	1.44±0.07e	0.13±0.01c	0.20±0.02c
T <sub>3</sub>	1.76±0.01b	1.99±0.00a	0.25±0.02d	0.27±0.01de	1.62±0.01e	2.01±0.00ef	1.80±0.01b	1.75±0.02a	0.11±0.01cd	0.19±0.03c
T <sub>4</sub>	1.43±0.02d	1.70±0.02c	0.28±0.02cd	0.29±0.01cd	1.59±0.01e	2.00±0.01f	1.68±0.02c	1.44±0.03e	0.12±0.00cd	0.20±0.01c
T <sub>5</sub>	1.56±0.01c	1.77±0.00c	0.34±0.02b	0.33±0.01b	1.80±0.01b	2.09±0.01c	1.94±0.01a	1.53±0.01de	0.17±0.01b	0.27±0.01ab
T <sub>6</sub>	1.74±0.03b	1.88±0.01b	0.43±0.01a	0.42±0.01a	1.87±0.01a	2.13±0.00a	1.99±0.00a	1.72±0.02ab	0.21±0.01a	0.31±0.00a
T <sub>7</sub>	1.84±0.01a	2.04±0.01a	0.32±0.01bc	0.31±0.01bc	1.80±0.00b	2.11±0.00b	1.85±0.01b	1.65±0.02bc	0.18±0.00b	0.29±0.00ab
T <sub>8</sub>	1.62±0.01c	1.86±0.02b	0.32±0.02bc	0.31±0.02bc	1.73±0.00c	2.02±0.01e	1.82±0.00b	1.61±0.01cd	0.17±0.01b	0.26±0.00b

Mean ± Standard error, n = 2; values followed by same letter in a column were not significantly different (p ≤ 0.05).

**Table 3.** Response of Cleopatra mandarin to microbial inoculants on leaf micro nutrient content (ppm).

	Fe		Cu		Mn		Zn	
	WS	WW	WS	WW	WS	WW	WS	WW
T <sub>1</sub>	26.93±0.55g	37.44±0.12g	6.98±0.06d	8.29±0.03e	70.64±0.43g	77.65±0.27f	8.45±0.58e	12.71±0.35d
T <sub>2</sub>	37.56±0.07e	40.91±0.36f	8.05±0.03c	9.18±0.01d	78.18±0.95f	80.94±0.04e	11.99±1.58d	18.18±1.08bc
T <sub>3</sub>	40.15±0.02d	47.02±0.21d	7.95±0.04c	9.17±0.03d	79.63±0.04e	81.28±0.01e	12.54±1.33d	17.59±0.91c
T <sub>4</sub>	35.15±0.02f	42.08±0.27e	7.82±0.17c	8.98±0.05d	81.45±0.13d	83.05±0.04d	15.04±1.05bcd	19.03±1.16bc
T <sub>5</sub>	46.51±0.18c	50.71±0.24b	9.44±0.20b	10.50±0.21c	87.56±0.25c	89.74±0.08c	17.25±1.71bc	20.48±1.30bc
T <sub>6</sub>	49.96±0.18a	52.74±0.03a	10.14±0.01a	11.98±0.01a	90.22±0.19a	91.58±0.18a	21.09±0.13a	27.31±0.70a
T <sub>7</sub>	47.46±0.12b	50.76±0.05b	9.66±0.12b	10.93±0.03b	89.15±0.01ab	90.88±0.06b	17.89±1.11ab	21.39±1.31b
T <sub>8</sub>	46.64±0.03c	49.71±0.03c	10.30±0.30a	11.12±0.26b	88.68±0.06bc	90.84±0.11b	14.46±0.44cd	20.69±1.51bc

Mean ± Standard error, n = 2; values followed by same letter in a column were not significantly different (p ≤ 0.05).

statistically at par with that of rhizosphere of seedlings co-inoculated with *G. intraradices* and *Providencia* sp. (AW 5) under WW condition (86.51%).

## DISCUSSION

Colonization of roots by AMF and the subsequent benefits derived by a host plant depend initially on the survival of AMF propagules, particularly spores. The higher *G. intraradices* colonization in roots of Cleopatra mandarin treated with PSB might be due to improved AMF interaction with plant roots due to production of active metabolites such as vitamins, amino acids and indole-3-acetic acid by the bacteria (Vivas et al., 2003), resulting in increased germination of fungal spores and rapid AMF establishment in soil (Toljander et al., 2006).

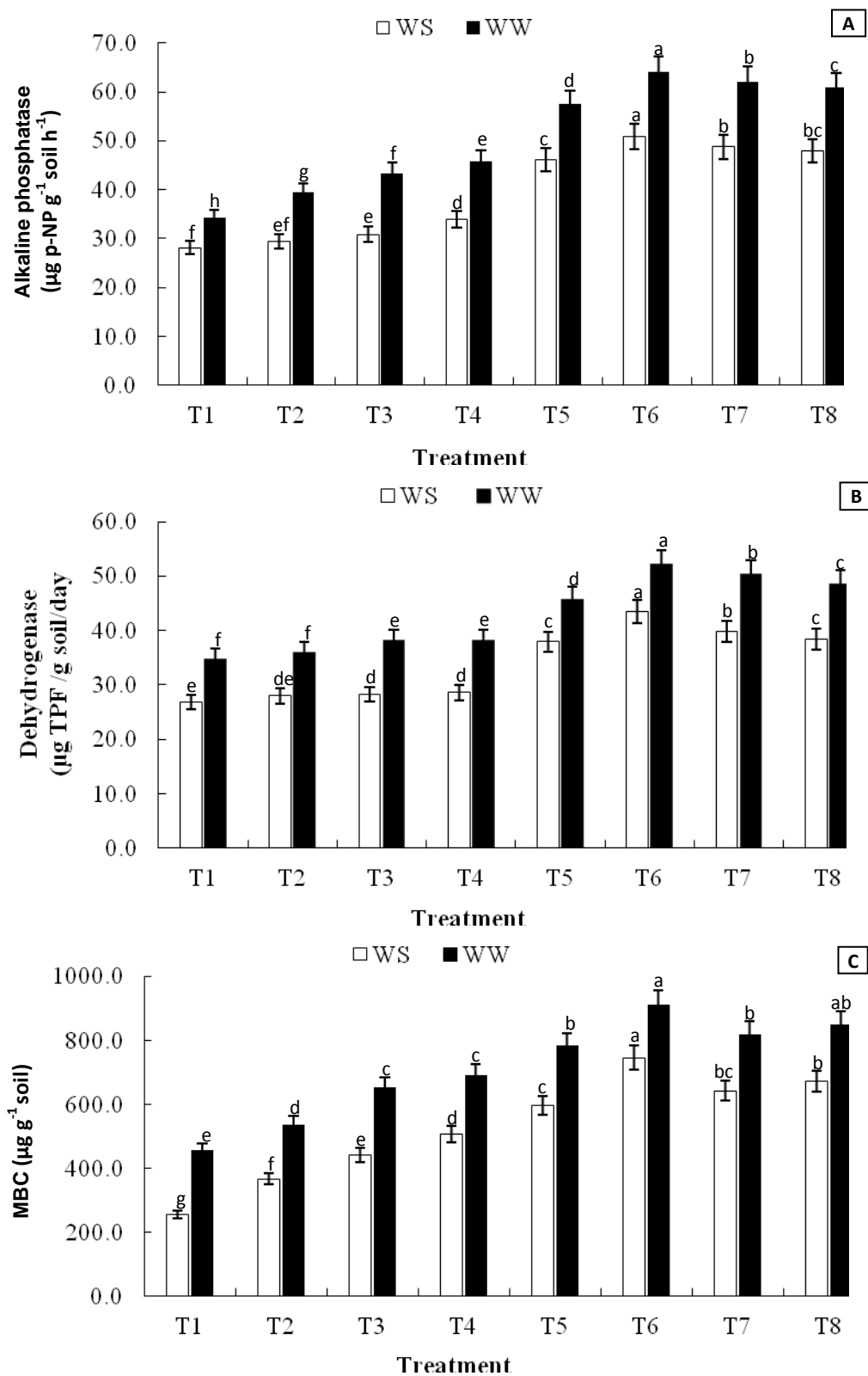
Drought had adverse effect on biomass production in Cleopatra mandarin. The synergistic effect of PSB with *G. intraradices* for increase in both shoot and root weight indicated its compatible interaction with AMF. This increase in plant growth has been attributed to the stimulation of activity of AM fungal mycelium in the rhizosphere by PSB (Marulanda et al., 2003), resulting in improving root-fungus interaction, thereby increasing the

hyphal efficiency for exploring greater volume of soil for water and nutrient uptake (Allen, 2011).

The AMF inoculation can lower the H<sub>2</sub>O<sub>2</sub> accumulation in plants (Wu et al., 2006) and also enhance the production of SOD involved in catalyzing the conversion of free O<sub>2</sub><sup>-</sup> to O<sub>2</sub> (Huang et al., 2010). Thus, lower ROS levels in leaves of Cleopatra mandarin co-inoculated with AMF and MHB might be due to the fact that bacteria induced a higher increase in antioxidant enzyme activities in AM plants in response to stress, resulting in alleviating the negative effect of stress (Kohler et al., 2009).

The AMF possess several special genes encoding for antioxidant enzymes, whose expression patterns can regulate the activities of antioxidant enzymes (Wu and Zou, 2009). In the present study, the enhanced antioxidant activity in AMF and MHB co-inoculated seedlings supports the view that increased enzyme activities could be involved in the beneficial effects of microbial inoculation on the performance of plants grown under semi-arid conditions (Alguacil et al., 2003), indicating better plant protection against the drought stress (Azcón et al., 2013).

The plants inoculated with AMF can accumulate antioxidants to counteract ROS under any environmental stress (Kaya et al., 2009). The synergistic effect of MHB



**Figure 4.** Influence of microbial inoculants on (A) alkaline phosphatase; (B) dehydrogenase; (C) microbial biomass carbon (MBC) in rhizosphere of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at  $p \leq 0.05$ ,  $n = 2$  (Web Agri Stat Package 2.0).

with AMF leading to higher antioxidant level might help the host plant in dissipating the photo-synthetically produced electrons and in alleviating oxidative damage.

The higher foliar N content in Cleopatra mandarin co-inoculated with *G. intraradices* and *A. brasilense* could be attributed to the increase in N-assimilating enzymes, such as nitrate reductase in the shoots of AMF colonised plants (Caravaca et al., 2005). The higher level of P in seedlings co-inoculated with *G. intraradices* and PSB might be due to release of phosphate ions from sparingly soluble inorganic and organic P compounds in soil, thereby contributing to increased soil phosphate pool available for the extraradical AM fungal hyphae to pass on to the plant (Artursson et al., 2006). The enhanced acquisition of other mineral nutrients (K, Ca, Mg, Fe, Cu, Mn and Zn), in co-inoculated microbial treatment, could be attributed to the greater absorption of the surface area provided by extensive fungal hyphae (Navarro et al., 2011). The acquisition of P and Ca by AM plants were recorded lesser under WW than WS condition, which could be attributed to improved efficiency of fungal hyphae in transfer of nutrients especially phosphorus and calcium which are immobile in soil and plant, respectively, from nutrient depletion zone to root cortex. Bryla and Duniway (1997) reported that AM fungal infection might be less beneficial for P transfer in plants under well watered condition than under drought condition.

Mycorrhizal symbionts are an integral part of the rhizosphere microflora, and thus contribute to the dynamic equilibrium of the rhizosphere. The increased alkaline phosphatase and dehydrogenase activities and MBC content in seedlings co-inoculated with *G. intraradices* and PSB could be due to increase in the rhizosphere microbial population as a consequence of the inoculation treatments (Aseri and Tarafdar, 2006).

From the above results, it could be concluded that co-inoculation of *G. intraradices* and PSB could be done in nursery in citrus propagation, which was found to synergistically interact to improve growth and performance of Cleopatra mandarin seedlings, much better than other microbial combinations under drought stress.

### Conflict of interests

The authors did not declare any conflict of interest.

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## Full Length Research Paper

# AmpC and metallo beta-lactamases producing Gram negative bacteria in patients with hematological malignancy

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Resistance to broad spectrum  $\beta$ -lactams mediated by AmpC and metallo beta-lactamases (MBLs) enzymes is a rising problem worldwide. The wide dissemination of Gram negative bacteria harboring these enzymes represents a significant clinical threat during the last decade, which is mainly due to treatment failure and restriction of therapeutic options. This problem should be really estimated in our locality with special emphasis on immunocompromised patients. The aim of this study was to isolate Gram negative bacteria from different sites of infection among patients with hematological malignancy, and to examine those isolates for AmpC and MBLs production by phenotypic and genotypic methods. Seventy four Gram negative bacterial strains were isolated from 387 clinical samples collected from different infection sites. Those isolates were screened for the presence of AmpC and MBLs by modified three dimensional test and Imipenem–EDTA combined disc test, respectively. Multiplex PCR was done as a confirmatory step for detection of AmpC and MBLs production by these isolates. *Pseudomonas aeruginosa* was the most common isolated Gram negative strain that was found to be positive for AmpC and MBL production. DHA gene was the most frequently detected AmpC  $\beta$ -lactamase gene, whereas VIM was the only detected MBL gene among the Gram negative bacterial isolates by multiplex PCR. The strong association found between AmpC production and MBL gene carriage is alarming which necessitate continuous surveillance of such resistance mechanisms among the Gram negative bacteria, especially in patients with hematological malignancy.

**Key words:** AmpC, metallo beta-lactamase (MBL), multiplex polymerase chain reaction, *Pseudomonas aeruginosa*.

## INTRODUCTION

$\beta$ -Lactamases are important components of the anti-microbial resistance in Gram negative bacteria. They are identified in these pathogens as well as their biochemical

properties (Zavasci et al., 2010). These enzymes inactivate cephalosporins and penicillins by hydrolyzing the amide bond of the  $\beta$ -lactam ring. Molecular class C or AmpC

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primarily hydrolyses cepheems (cephamycins and cephalosporins) and also hydrolyze penicillins and aztreonam. These enzymes are resistant to the currently available  $\beta$ -lactamase inhibitors such as clavulanate, sulbactam and tazobactam (Philippon et al., 2002). With rare exceptions, the hydrolysis of cephamycins, such as cefoxitin and cefotetan, is a property that can help to distinguish AmpCs from extended-spectrum beta lactamase (ESBLs).

High level production of AmpC by Gram negative bacteria may cause resistance to the first, second and third-generation cephalosporins, penicillins, cephamycins and  $\beta$ -lactamase inhibitor combination. Higher level AmpC production may occur as a consequence of mutation or when the organism is exposed to an inducing agent. Cephamycins (e.g. cefotetan and cefoxitin), ampicillin, and carbapenem are good inducers (Moland et al., 2008).

Carbapenamases are  $\beta$ -lactamases, which include serine- $\beta$ - lactamases (OXA, KPC, GES, etc.) and MBLs. The latter require metal ion zinc for their activity, which is inhibited by metal chelators like EDTA and thiol-based compounds but not by tazobactam, sulbactam and clavulanic acid. MBL producing Gram negative bacteria are typically resistant to aminoglycosides and fluoroquinolones, further compromising therapeutic options (Uma Karthika et al., 2009).

Among several types of MBL genes described throughout the world, *bla*-VIM, *bla*-IMP and *bla*-NDM are the most common (Amudhan et al., 2011). The genes responsible for MBL production may be chromosomal or plasmid mediated and poses a threat of horizontal transfer among other Gram-negative bacteria (Varaiya et al., 2008).

Infections remain a common complication in patients with hematological malignancies. These patients are at higher risk of infections not only because of the malignancy itself but also because of neutropenia induced by intensive chemotherapeutic therapy that may be followed by hematopoietic stem cell transplantation, and the cytotoxic effect on the cells that line the alimentary tract (Crawford et al., 2004).

It was demonstrated that 60% of the bacteraemias in neutropenic patients were caused by Gram negative bacterial infections (Chen et al., 2010). Moreover, the increased rates of drug resistant Gram-negative pathogens which was documented globally (Bhusal et al., 2011), including ESBLs, AmpC and carbapenemase producing Gram-negative bacteria isolates (Freifeld et al., 2011) and despite of the improved survival rate during the last decades, patients with haematological malignancies are still at high risk of infectious complications. Bacteraemia caused by beta-lactamase producing Gram negative bacteria are serious complications and the use of prophylaxis may lead to a higher prevalence of more resistant strains (Nørgaard, 2012). So the aim of this study was to detect Gram negative bacterial infection

among these patients and to examine the bacterial isolates for AmpC and MBLs production by phenotypic and genotypic methods.

## MATERIALS AND METHODS

### Study design

Descriptive cross sectional study was carried out on 373 patients with hematological malignancy over a period of six months from first of June to the end of November, 2013. All patients, enrolled in this study, were admitted in the Oncology Centre of Mansoura University Hospitals (MUHs).

### Clinical samples

387 clinical samples collected from different infection sites included 139 urine, 95 sputum, 86 blood and 67 wound swabs.

### Microbiological studies

Samples were processed in Microbiology Diagnostic and Infection Control Unit (MDICU) in Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University. The collected blood, sputum and wound specimens were cultivated on blood agar, macConkey's agar and chocolate agar media, whereas urine specimens were cultivated on CLED media. Gram negative bacterial isolates was identified by Gram stained films, colony morphology and different biochemical reactions.

### Antibiotic susceptibility testing

Antimicrobial susceptibility was tested for the identified Gram negative isolates according to CLSI recommendations. The diffusion method on Mueller-Hinton agar (MHA; Bio-Rad, Marnes-La-Coquette, France) was used to test susceptibility to Ampicilline, Amoxicilline/Clavulanic acid, Cefotaxime, Azteronam, Imipenem, Pipracilline, pipracilline/Tazobzctam, Amikacin, Ciprofloxacin, Gentamicin, Ceftazidime (Koneman et al., 1997).

### Phenotypic detection of AmpC by modified three dimensional test (Taneja et al., 2008)

Crude enzyme extract was prepared by repeated freezing and thawing of the bacterial pellet of each Gram negative bacterial isolate (five to seven times). Lawn culture of *Escherichia coli* ATCC 25922 was prepared on Muller Hinton plates and Cefoxitin (30  $\mu$ g) disk were placed on the plates. Linear slits (3 cm) were cut using sterile surgical blade, 3 mm away from cefoxitin disk. At the other end of the slit, a small circular well was made and 30 to 40  $\mu$ l of the enzyme extract was loaded in the well. Then, the plates were incubated at 37°C for 24 h. Enhanced growth of the surface organism at the point where the slit inserted the zone of inhibition of cefoxitin was considered a positive modified three dimensional test. Isolates showing clear distortion of the zone of inhibition of cefoxitin were considered as AmpC producers.

### Phenotypic detection of MBL by Imipenem-EDTA combined disc method (Yong et al., 2002):

A 0.5 M EDTA solution was prepared by dissolving 186.1 g of

**Table 1.** Primers used in multiplex PCR and PCR mapping in this study.

Primer	Sequence 5'-3'	Amplicon size (bp)	Reference	
MultiCaseACC_for	CACCTCCAGCGACTTGTTAC	346	Dallenne et al., 2010	
MultiCaseACC_rev	GTTAGCCAGCATCACGATCC			
MultiCaseFOX_for	CTACAGTGCGGGTGGTTT	162		
MultiCaseFOX_rev	CTATTTGCGGCCAGGTGA			
MultiCaseMOX_for	GCAACAACGACAATCCATCCT	895		
MultiCaseMOX_rev	GGGATAGGCGTAACTCTCCCAA			
MultiCaseDHA_for	TGATGGCACAGCAGGATATTC	997		
MultiCaseDHA_rev	GCTTTGACTCTTTTCGGTATTCG			
MultiCaseCIT_for	CGAAGAGGCAATGACCAGAC	538		
MultiCaseCIT_rev	ACGGACAGGGTTAGGATAGY <sup>b</sup>			
MultiCaseEBC_for	CGGTAAAGCCGATGTTGCG	683		
MultiCaseEBC_rev	AGCCTAACCCCTGATACA			
MultiIMP_for	TTGACACTCCATTTACDG <sup>b</sup>	139		
MultiIMP_rev	GATYGAGAATTAAGCCACYCT <sup>b</sup>			
MultiVIM_for	GATGGTGTGTTGGTCGCATA	390		Ellington et al., 2007
MultiVIM_rev	CGAATGCGCAGCACCAG			
MultiNDM_for	GGTTTGGCGATCTGGTTTTC	621	Anoar et al., 2014	
MultiNDM_rev	CGGAATGGCTCATCACGATC			

<sup>b</sup>Y = T or C; D = A or G or T.

disodium EDTA•2H<sub>2</sub>O in 1000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. One disc of imipenem (10 µg) alone and one with imipenem (10 µg) in combination with EDTA solution were placed at a distance of 20 mm, from center to center, on a Muller Hinton agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards of each Gram negative bacterial isolate. The plates are incubated overnight at 35°C. The MBL producing strains showed a variation greater than 7 mm between the inhibition zone around imipenem discs alone and the inhibition zone around imipenem+ EDTA discs, (the imipenem + EDTA discs shows larger zone of inhibition than imipenem disc alone in MBL positive strains).

#### Detection of plasmid encoded AmpC and MBL genes by multiplex PCR

##### Design of group-specific primers for multiplex PCR assays

Two multiplex PCRs were designed in this study: a plasmid-mediated AmpC β-lactamase gene multiplex PCR including six family-specific AmpC genes carried on the plasmids, which are ACC, FOX, MOX, DHA, CIT and EBC (Perez-Perez and Hanson, 2002); and MBL gene multiplex PCRs, including *bla*<sub>VIM1</sub>, *bla*<sub>IMP</sub> and *bla*<sub>NDM</sub> genes (Amudhan et al., 2011). Group-specific primers were designed to amplify internal fragments of several sizes (Table 1).

##### Multiplex PCR technique (Dallenne et al., 2010)

Rapid DNA preparation was performed from one heated colony in a total volume of 100 mL of distilled water (95°C for 10 min) followed by a centrifugation step of the cell suspension. Total DNA (2 mL) was subjected to each multiplex PCR in a 50 mL reaction mixture containing 1×PCR buffer (10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM MgCl<sub>2</sub>), 200 mM concentration of each deoxynucleotide triphosphate, a variable concentration of specific-group primers

(Table 1) and 1 U of Taq polymerase (Fermentas). Amplification was carried out as follows: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 7 min. For the MBL genes multiplex PCR assays, the annealing temperature was optimal at 55°C for amplification of *bla*<sub>VIM1</sub>, *bla*<sub>IMP</sub> and *bla*<sub>NDM</sub> genes. Amplicons were visualized after running at 100 V for 1 h on a 2% agarose gel containing ethidium bromide. A 100 bp DNA ladder (#SMO323 marker "Fermentas") was used as a size marker.

#### Data analysis

Data were entered and statistically analyzed using Statistical Package of Social Science (SPSS) software version 17. Qualitative data were described as numbers and percentages.

## RESULTS

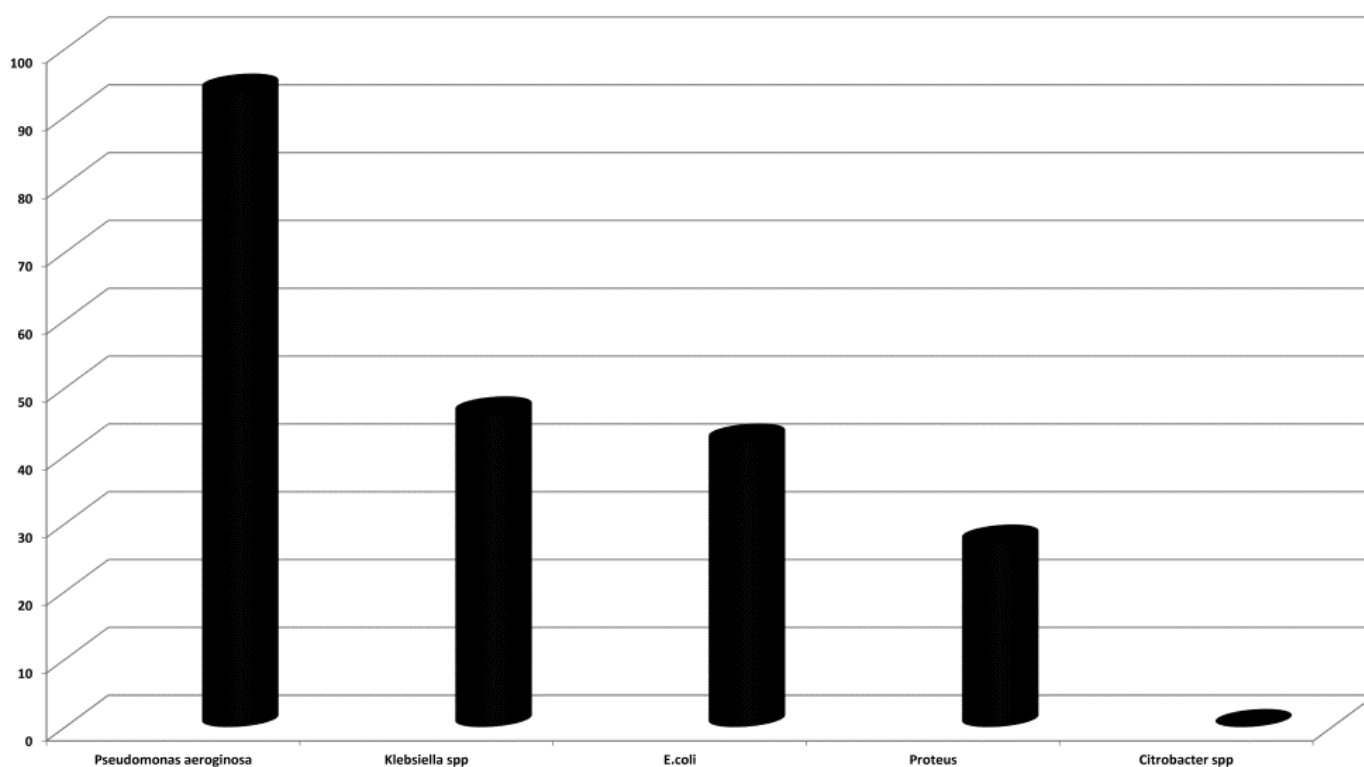
Out of the 387 clinical samples, 74 Gram negative bacterial isolates were detected, including 26 *Klebsiella* spp., 19 *E. coli*, 16 *Pseudomonas aeruginosa*, 11 *Proteus* spp. and 2 *Citrobacter* spp. Urine was the most common source of the isolated Gram negative bacteria (42%), followed by sputum (28%), wound (18%) and blood (12%).

Percentage of resistance exhibited by the 74 Gram negative bacterial isolates to various antimicrobial agents is shown in (Table 2). AmpC was detected in 38 (51.3%) of the 74 Gram negative bacterial isolates which were studied in this research. Among the 16 *Pseudomonas aeruginosa* isolates, 15 (93.7%) isolates were found to be

**Table 2.** Antibiotic resistance pattern of the Gram negative bacterial isolates.

Bacterial isolates	<i>Pseudomonas aeruginosa</i>		<i>Klebsiella</i> spp		<i>E. coli</i>		<i>Proteus</i> spp		<i>Citrobacter</i> spp	
	R	%	R	%	R	%	R	%	R	%
Ampicilline	16	100	24	92.3	18	94.7	11	100	2	100
Amoxicilline/Clavulinic acid	12	75	16	61.5	16	84.2	8	72.7	1	50
Azteronam	7	43.7	9	34.6	8	42.1	9	81.8	1	50
Cefotaxime	15	93.7	19	73	10	52.6	9	81.8	2	100
Imipenem	9	56.2	8	30.7	11	57.9	6	54.5	1	50
Pipracilline	11	68.7	7	26.9	17	89.4	11	100	2	100
pipracilline/Tazobzctam	8	50	6	23	12	63.1	7	63.6	0	0
Amikacin	14	87.5	14	53.8	13	68.4	8	72.7	1	50
Ciprofloxacin	12	75	22	84.6	14	73.6	10	90.9	2	100
Gentamicin	15	93.7	17	65.3	14	73.6	9	81.8	2	100
Ceftazidime	14	87.5	20	76.9	11	57.9	11	100	2	100

spp: Species, R: Resistant, %: percentage

**Figure 1.** Percentage of AmpC production among the Gram negative bacterial isolates.

positive for AmpC production by modified 3 dimensional test. Whereas 12 (46.1%) isolates of *Klebsiella*, 8 (42.1%) isolates of *E. coli*, 3 (27.2%) isolates of *Proteus* spp. and none of *Citrobacter* spp. were found to be positive by the same test, (Figures 1 and 2).

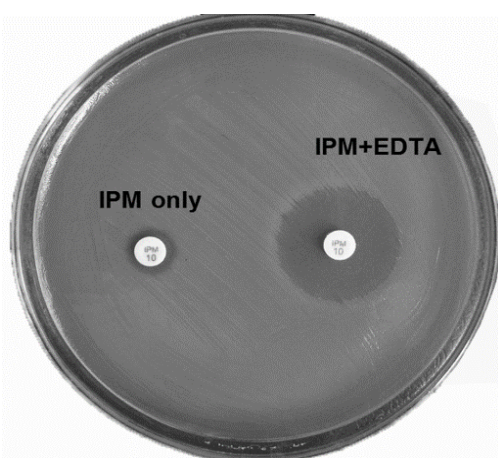
MBL was detected in 20 (27%) of the studied 74 Gram

negative bacterial isolates. It was present in 11 (68.7%), 7 (26.9%), 2 (18.1%) of *Pseudomonas aeruginosa*, *Klebsiella* spp. And *Proteus* spp. respectively by imipenem-EDTA disc method, (Figure 3), whereas none of *E. coli* and *Citrobacter* spp. were found to be positive by the same test.





**Figure 2.** Positive modified three dimensional test for one of the isolated Gram negative bacteria. Enhanced growth of the surface organism, *E. coli* ATCC25922, is seen near the agar slit that contain extract of the Gram negative bacterial isolate.



**Figure 3.** Positive imipenem-EDTA combined disc test for one of the isolated Gram negative bacteria. The imipenem + EDTA discs shows larger zone of inhibition than imipenem disc alone.

As regarding the result of multiplex PCR assay (Figure 4), that was performed for the strains that were positive for AmpC and MBL by phenotypic detection methods, it was found that, plasmid-mediated AmpC  $\beta$ -lactamase genes were detected in 31 (81.5%) of the 38 AmpC positive Gram negative bacterial isolates, whereas only 12 (60%) of the 20 MBL producing isolates were found to be positive for MBLs genes, and we observed 5 isolates that were positive for both AmpC  $\beta$ -lactamase and MBLs genes.

DHA genes was the most frequently observed plasmid mediated AmpC  $\beta$ -lactamases (found in 23 isolates, "60.5%"); whereas 5 isolates, "13.1%" and only 3,

isolates "7.8%" were found to be positive for CIT and ACC genes group, respectively.

VIM genes were the only detected MBLs gene among the studied bacterial isolates by multiplex PCR, whereas, no isolates were found to be positive for IMP or NDM genes.

## DISCUSSION

The clear role of Gram negative bacteria in the production of various  $\beta$ -lactamase enzymes have been reported with an increasing frequency as an important hospital problem because they are associated with high morbidity and mortality rates (Itokazu et al., 1996).

Initially these enzymes were commonly found in *Klebsiella* species and *E. coli* (Mathur et al., 2002), but now they are produced by all the members of *Enterobacteriaceae* and other Gram negative bacteria (Kumar et al., 2006). These enzymes are capable of hydrolyzing broad spectrum cephalosporins and monobactams and inactive against imipenem and cephamycins (Albertini, 2002).

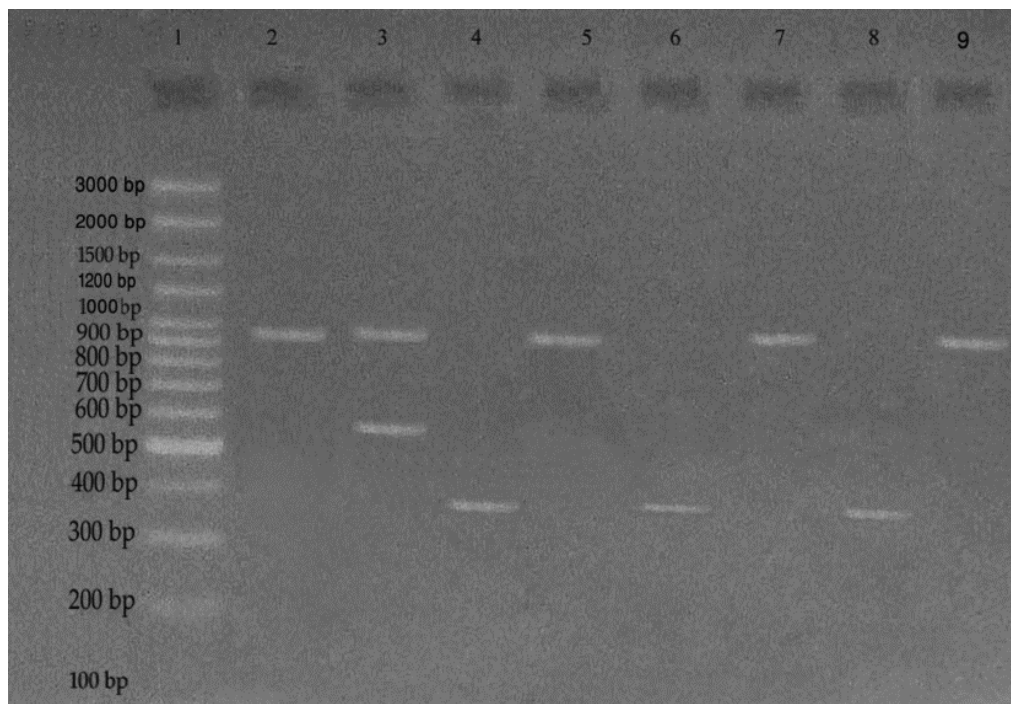
It was documented that bacteraemias in neutropenic patients were mostly caused by Gram negative infections (Chen et al., 2010) and a similar pattern was demonstrated by Gupta et al. (2010) in a study from India. Gram-negative bacterial strains (*E. coli*, *Klebsiella* spp. and *P. aeruginosa*) were the predominant infectious bacteria in neutropenic cancer patients in the 1970s and early 1980s (Carratala and Gudiol, 2000). These findings confirm the role of Gram negative bacteria as a source of different types of enzymes that induce high degree of resistance, particularly AmpC and MBLs in those patients that could badly affect their outcome and survival.

AmpC  $\beta$ -lactamases are cephalosporinases which are encoded on chromosomes of many of the *Enterobacteriaceae* and a few other organisms, and they mediate resistance to cephalothin, cefoxitin, cefazolin, most of the penicillins and  $\beta$ -lactamase inhibitor. AmpC enzymes are inducible and can be expressed at high levels by mutation in different types of Gram negative bacteria. Over expression can induce resistance to broad spectrum cephalosporins (Black et al., 2005).

In this study, 51.3% of the Gram negative isolates were AmpC producer and *P. aeruginosa* was the predominant AmpC producing strain. These results were in agreement with Altun et al. (2013), who found that all the studied *P. aeruginosa* isolates were AmpC producer. On the other hand, lower percentage of AmpC production among *P. aeruginosa* isolates (72.4 and 59.4%) was reported by Abd El-Baky et al. (2013) and Upadhyay et al. (2010) respectively.

The discrepancy among different studies could be attributed to different localities where each one has its own pattern of pathogens distribution and resistance.

MBL is a group of carbapenem hydrolysing  $\beta$ -lactamase (Chu et al., 2001). They have been reported in



**Figure 4.** The results of the multiplex PCR assay of the Gram negative bacterial isolates. The figure shows the multiplex PCR results with primers specific for AmpC and MBLs genes. Lane 1 shows molecular size marker #SMO323. Lanes 2, 3, 5, 7 and 9 shows bands of 997 base pair from positive strains that carry the DHA genes, whereas lanes 4, 6 and 8 shows bands of 390 base pair from positive strains that carry the VIM genes and lane 3 shows band of 538 base pair of the CIT gene.

multidrug resistance pathogens like *P. aeruginosa* and *Acinetobacter* species. The MBLs are inhibited *in vitro* by  $\text{CuCl}_3$ ,  $\text{FeCl}_3$ , EDTA and thiol compounds, but not by  $\beta$ -lactamase inhibitors like Clavulanic acid, Sulbactam or Tazobactam (Goossens, 2000). Detection of MBL production in Gram negative bacteria from patients with hematological malignancy has tremendous therapeutic consequences, as the treatment option for such isolates are aztreonam or potentially toxic polymyxin B and colistin.

In the present study, MBL was detected by imipenem-EDTA disc method in 20% of the studied isolates and *P. aeruginosa* was also the predominant MBL producing strain (68.7%). Similarly, Noyal et al. (2009) stated that 50% of the studied *P. aeruginosa* strains were positive for MBL by the same test and it was the most common MBL producing strain among his studied Gram negative bacterial isolates. On the other hand, our results are dissimilar with that of Mishra et al. (2012) and Altun et al. (2013), who found that *Acinetobacter* were the most common MBLs producing strains in their study, and reported a higher incidence of MBL production than *P. aeruginosa* strains.

*P. aeruginosa* may be intrinsically resistant or have acquired resistance to antibiotics due to permeability barrier of the cell surface, multidrug efflux pumps and production of  $\beta$ -lactamases (AmpC  $\beta$ -lactamase, extended

spectrum  $\beta$ -lactamases and metallo- $\beta$ -lactamases) (Pai et al., 2004). Multiple beta-lactamase producing *P. aeruginosa* can cause major therapeutic failure, and poses a significant clinical threat if remain undetected. Therefore, early identification of the infections due to these microorganisms is necessary as the appropriate treatment might reduce the spread of these resistant strains as well as the mortality in hospitalized patients. This emphasizes the need for the detection of isolates that produce these enzymes to avoid therapeutic failures and nosocomial outbreaks in hospitals (Kumar et al., 2012).

In our study, 93.7 and 68.7% of *P. aeruginosa* strains were positive for AmpC and MBL respectively. Carbapenems were the effective antibiotics for MDR Gram-negative bacteria infections, especially in high-risk hospital settings, particularly, *P. aeruginosa* (Deshpande et al., 2010). Therefore, when administering empirical treatment in patients with hospital-acquired infections due to *Pseudomonas* spp., if patients do not respond to carbapenem therapy, MBL should be considered.

Multiplex PCR is one of the most important diagnostic methods that is used to determine the most frequent widespread  $\beta$ -lactamase genes encoding plasmid-mediated AmpC and MBLs, PCR is a fast, low-cost and It helps in monitoring the emergence of those genes and their spread, and it could also be used in epidemiological

surveys.

DHA genes was the most frequently observed plasmid mediated AmpC  $\beta$ -lactamases (found to be 60.5%) of the studied AmpC positive strains, whereas VIM genes was the only detected MBLs genes among the studied bacterial isolates by multiplex PCR. Our results were consistent with the finding of Dallenne et al. (2010), who reported that, DHA enzymes were the most frequently observed AmpC  $\beta$ -lactamases among their studied isolates, but in their study, they did not find any of the MBLs genes that were targeted. Parallel to our finding, Chakraborty et al. (2011), found that most of the studied strains were found to amplify the VIM primer and no IMP genes got amplified.

On the other hand, our results were dissimilar with that of Manoharan et al. (2012), who mentioned that CIT and FOX were the most frequently detected AmpC  $\beta$ -lactamases genes, whereas DHA was the least frequently detected one. Also contrary to our findings, Anoar et al. (2014), observed IMP as the most frequently found MBL gene among their studied isolates and it recorded a higher percentage of detection than VIM and NDM genes.

In our research, the percent of strains that carried AmpC and MBL genes was higher than those reported in previous studies. The reasons may be an overall increase in the extent of acquirement of AmpC and MBLs genes among *P. aeruginosa*, which was highly isolated in this study plus its high resistance pattern which is characteristic in our locality. Moreover, the location of AmpC and MBL genes on plasmide and on class I integron respectively, can therefore easily transfer between the Gram negative bacterial isolates particularly, *P. aeruginosa* strains (Cornaglia et al., 2000).

Of the MBL phenotypic-positive isolates, 8 did not carry the MBL genes. It may be possible that these isolates harbor other MBL genes that were not detected in this study. Another possibility may be the susceptibility of some bacterial strains to EDTA, which can affect bacterial membrane permeability, leading to false positive results for the MBL phenotypic tests (Aktas and Kayacan, 2008). Also, sizeable number of isolates were not positive for AmpC production by the multiplex PCR, but they were phenotypically positive, this warrants further molecular investigation into the other mechanisms of resistance and their laboratory detection.

Although the modified three dimensional test and Imipenem–EDTA combined disc test reliably detected AmpC and MBL when compared with the PCR in the present study, clinical laboratories interested in distinguishing AmpC and MBL mediated resistance from other  $\beta$ -lactamase resistance mechanisms will need to use combination of phenotypic and molecular identification methods, and we thought that the multiplex PCR technique described in this study will be an important tool for the detection of AmpC  $\beta$ -lactamases and MBL genes in Gram-negative bacteria.

## Conclusion

Despite the discovery of AmpC and MBL at least a decade ago, there remains a low level of awareness of their medical importance and many clinical laboratories have problems in detecting them. On the other hand, the strong association found between AmpC production and MBL gene carriage is alarming and would limit the choice of antibiotic treatment even more. So, this study findings indicate the necessity for continuous surveillance of such resistance mechanisms among the Gram negative bacteria and evolve preventive measures aimed at reducing their spread among patients with hematological malignancy.

## Conflict of interests

The author(s) did not declare any conflict of interest.

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## Full Length Research Paper

# Antibacterial activity of two extracts from *Rubus fruticosus* L. against resistant pathogens and their antioxidant potential

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One of the most serious threats to public health across the world is the spread of antibiotics resistant pathogens. This situation obliges researchers from all over the world to focus on how they can overcome this obstacle mainly by searching or synthesizing new molecules to neutralize microbes and to overcome the phenomenon of resistance. One of the main reservoirs of potentially active molecules is the plantae kingdom. In this work, we focused on *Rubus fruticosus* L., a medicinal species which is used traditionally in Algeria to heal respiratory infections. Both of chemical composition and biological activities of essential oils and flavonoids extract were explored. GC-MS analysis of essential oil reveals the Linceol as the most abundant component (20.22%). HPLC applied on flavonoids extract shows the presence of the gallic acid, galocatechin, protocatechuic acid, catechin, caffeic acid, rutin, ellagic acid and myricetin. Tests of antioxidant capacity using the DPPH reveals an inhibiting effect which is more important with the flavonoid extract. Otherwise, resistant strains to conventional antibiotics seem to be sensitive to the flavonoids of this species with MICs varying from 2.36 to 75.54 mg/ml. This study indicates that *R. fruticosus* L. has an important antimicrobial effect, which justifies its potential use in infectious diseases. For many reasons, this species remains poorly studied, further refined studies on its pure secondary metabolites are needed in the perspective of identifying new antimicrobial molecules from this plant.

**Key words:** *Rubus fruticosus* L., essential oil, flavonoids, antimicrobial activity, resistant respiratory pathogens, antioxidant activity.

## INTRODUCTION

Respiratory infections are considered as a major risk for human health, beside emerging respiratory infections

such as severe acute respiratory syndrome (SARS), avian influenza and Hantavirus pulmonary syndrome,

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some respiratory infections are considered as eradicated or nearly, reappear with serious consequences: every year more than 8 million cases of severe respiratory infections are reported causing 3.9 million death (Sansone and Orth, 2006).

Despite the fact that researchers developed an important arsenal to face these infections and others, emerging resistances to antibiotics come to complicate the situation, this resistance implies various mechanisms such as modifying or synthesizing new targets, enzymatic modification, increasing efflux of antibiotics or others, the result is an under activity or non-activity of an important number of antibiotics used nowadays.

Many solutions were suggested to face this situation, and one of the most promising options is trying to extract from the plants new molecules that can be safe and effective in healing such diseases, the number of medicinal plants are estimated to be between 40000 and 70000 which are very diverse source of bioactive molecules and about 80% of world population is currently using these plants (Ramawat, 2009).

The interest in using these plants by population and also in research and academic world is increasing; new journals and reference books are published to enable researchers to find evidence based knowledge. *Rubus fruticosus* L. (Rosaceae) is a shrub well known for its fruit, called blackberries which are marketed all over the world for its delicious taste and high nutritive value. The shrub may be original from Armenia and is nowadays spreading in Europe, Asia, Oceania, northern and southern America and northern Africa (Hummer and Janick, 2007; Swanston-Flatt et al., 1990; Zia-Ul-Haq et al., 2014).

This plant is understory vegetation, can reach 3 m in length and have spiny stems. It flourishes at the end of spring and the fruit ripens in autumn (Hummer and Janick, 2007). According to some authors, this plant can be a good remedy for bronchitis and respiratory infections (Blumenthal et al., 1998).

However, no studies are reported on the Algerian *R. fruticosus* L. Two studies in Iran and Pakistan were reported by the antimicrobial activity of this species, two others were interested with the antioxidant activity.

The purpose of this study was to highlight the antimicrobial activity of this plant on both reference strains and strains isolated from patients with respiratory infections and in the same time, the study shows the antioxidant properties of its flavonoids extract and essential oil.

## MATERIALS AND METHODS

### Biological material

The harvest of the aerial part of *R. fruticosus* was accomplished in spring in the area of Tizi Ouzou, in the north east of Algeria, about 80 km of Algiers, the capital, between 36° 43' 00" North and 4° 03' 00" East. To extract flavonoids, collected parts were dried in open air and sheltered from light, then transformed into powder using an

electrical crusher; this powder was maintained in tightly closed glass flasks.

The tested strains was either reference strains or isolated from patients diagnosed with respiratory infections and hospitalized in the department of infectious diseases in the hospital university of Tizi Ouzou, the identification was effected using biochemical galleries Api 20E, Api 20NE, or specific tests.

### Phytochemical screening

Phytochemical screening represents a set of colorimetric methods that can lead to detect the presence or lack of secondary metabolites and should be realized on plant powder or infusion. We searched mainly alkaloids, anthocyanins, saponosids, leuco-anthocyanins, total tannins, gallic tannins, catechin tannins, alkaloids, flavonoids, saponosids, irridoids, quinones, coumarins and mucilage (Harborne, 1998; Raaman, 2006).

### Extractions

The extraction of essential oil was effected by hydro distillation, to accomplish this, 100 g of fresh matter was soaked in a recipient of 1l filled with 600 ml of distilled water, the whole system was boiled for 3 h. Collected vapors were condensed by transiting a refrigerator and collected. Organic state was recovered by adding few milliliters of diethyl ether and the obtained oil was kept in a temperature between 0 and 4°C.

The extraction of flavonoids was realized according to Bruneton (1999) protocol. This extraction is based on the difference of solubility degrees of flavonoids in various organic solvents. This protocol includes two main steps: first, methanol is used to solubilize flavonoids and then washings using petroleum ether, diethyl ether, ethyl acetate and butanol are realized. After using butanol, the extract contains the most polar flavonoids.

### Characterization of the extracts

#### GC-MS of essential oils

Analysis of the chemical composition of the essential oil was carried out by chromatography and gas chromatography coupled with mass spectrometry, the apparatus was a GC Perkin Elmer 600, MS Perkin Elmer 600C, a column Rtx-VMS menu (60m long with a diameter of 250 µm). The carrier gas was helium with a flow rate of 1ml/min. 0.2 µl oil was used to analyze injection using a special syringe.

The temperature was 70°C for 1 min, ramp 3to 160°C, 1 min ramp 2°C/min to 230°C for 5 min. The injector temperature was 230°C.

#### Dosage of flavonoids using a spectrophotometry method

To dose flavonoids, we used the aluminum chloride colorimetric method (Baharun et al., 1996). Absorbance was read by a spectrophotometer (Optizen 2120 UV) at 430 nm. To determine the concentration of flavonoids in the extract, a calibration range was established using quercetin (1-25 µg/ml). The results of dosage are expressed in equivalent micrograms of quercetin for each gram of the extract.

#### High performance liquid chromatography

HPLC was used to achieve the quantitative analysis of flavonoids.

Column of silica was used as stationary phase (C18 reverse phase), this column measures 125 by 4.6 mm. A mixture of water/methanol/acetic acid (50:47:2.5) was used as a mobile phase, in isocratic system with a flow of 1 ml/min (Amarowicz et al., 2005). Extracts and standards were both analyzed with concentration of 0.5 mg/ml. The used volume was 20  $\mu$ l. Detection was achieved by a UV-Visible detector at 254 nm.

## Biological activities

### Antimicrobial activity

A steers machine which is a multiple seeding instrument was used to facilitate the study of the antimicrobial activity of essential oil and this is according to the recommendations of the French Society of Microbiology. After diluting essential oil in mediums: Mueller Hinton for bacteria or Sabouraud for fungi, they were let to solidify. Bacterial suspensions of 0.5 Mc Farland was put using spots. For assessing the flavonoid extract activity, plates of Mueller Hinton agar for bacteria and Sabouraud agar for fungi were inoculated by swabbing of standardized microbial suspension (0.5 Mc Farland), according to NCCLS recommendations (NCCLS, 2006), after that, on the agar, we placed discs of 6 mm diameter containing 10  $\mu$ l of extract with different concentrations.

As positive control, discs containing antibiotics was used and placed in the center of the plate. After incubating for 24 h at 37°C for bacteria and 48 h for fungi, we determined the antimicrobial activity of both extracts by measuring the MICs. Every test was performed in triplicate.

### Antioxidant activity

#### The DPPH test

To quantify the antioxidant activity of both flavonoids extract and essential oil, we used the method of Sanchez Moreno et al. (1998). For that, we prepared various concentrations of 0.1; 0.2; 0.4; 0.6; 0.8 and 1 mg/ml using a stock solution of both extracts obtained by dissolution in methanol. 1 ml from each one of the concentrations was added to 4 ml of DPPH solution whose concentration was 0.024 mg/ml, this was the preparation for assessing the activity of essential oil. For flavonoids, we mixed 25  $\mu$ l of each concentration with 975  $\mu$ l of the same solution of DPPH. Ascorbic acid was prepared using the same protocol. After 30 min, we measured the variation of absorbance using UV-visible spectrophotometer (Optizen 2120 UV), driven by a computing system in the wavelength of 517 nm. To express results in percentage, we used

$$\text{this formula: } I\% = 100 \times \frac{A_{\text{reference}} - A_{\text{test}}}{A_{\text{reference}}}$$

$A_{\text{reference}}$  is the absorbance of the control which contains only reactive.  $A_{\text{test}}$  is the absorbance of the extract.

### Statistical analysis of the data

Results of the antioxidant activity were expressed as mean  $\pm$  SD.

## RESULTS AND DISCUSSION

### Phytochemical screening

The phytochemical screening of *R. fruticosus L.* reveals abundant amount of total tannins, catechin tannins, gallic

**Table 1.** Phytochemical screening of *R. fruticosus*.

Test	Abundance
Total tannins	++
Catechin tannins	++
Gallic tannins	++
Flavonoids	++
Anthocyanins	-
Leuco- anthocyanins	-
Alkaloids	-
Senosids	+
Amidon	-
Saponosids	++
Irridoids	-
Glucosids	-
Mucilages	-
Coumarins	++
Quinons	-

++: abundance; +: presence; -: absence.

**Table 2.** Yields in % of flavonoids extracts.

Extract	%
Diethyl ether extract	21.96
Ethyl acetat extract	12.29
Butanolic extract	35.15
Flavonoids	30.6

tannins, flavonoids, senosids, saponosids, coumarins (Table 1).

This plant seems to have a high potential but there is no sufficient studies on its chemical composition and biological activities.

A study on the same species shows that it contains alkaloids, flavonoids, tannins, saponins and glycosides (Rameshwar et al., 2014). We found similar results with no glycosides, but the presence of other compounds such as sénosides and coumarins.

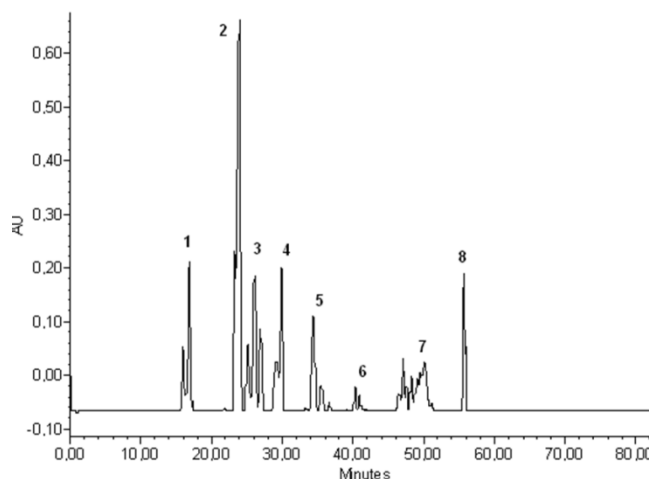
### Extractions

Using 100 g of dried matter, the yield was  $0.05 \pm 0.01\%$  of essential oil. We obtained lightly viscous oil, with a pale yellow coloration and a characteristic odor. The aqueous extract has a gelatinous aspect and brownish color. The yields of extraction using different solvents (diethyl ether, ethyl acetate, butanolic and aqueous) vary between 2.99 and 63.2% (Table 2).

Blackberry has about 30% of highly polar flavonoids (weak) against more than 60% butanol extract (the most abundant extract) in the plant of the present study. Total

**Table 3.** Retention time of the used standards.

Retention time (min)	Compound
17	Gallic acid
24.5	Gallocatechin
26.1	Protocatechic acid
30	Catechin
34.4	Caffeic acid
40.3	Rutin
50	Ellagic acid
55.6	Myricetin

**Figure 1.** Chromatogram of HPLC applied to flavonoids.

polyphenols of one Rosaceae plant contain a yield of about 140 mg/100 g of the material, although this performance is quite high, but we cannot show the percentage of each phenolic extract (Benvenuti et al., 2006).

### Characterization

#### Dosage of flavonoids

Content of flavonoids is expressed in equivalent mg of quercetin/ml of the extract of the whole plant. It was found to be 75.54 mg/ml. The content of *R. fruticosus* in flavonoids cannot be discussed because there is no published work on the chemical composition of this plant or another plant belonging to the same family, but by observing the averages of yields of extraction in many studies, 75.54% is a high yield and we it can be assumed that this extract is high in flavonoids.

#### High performance liquid chromatography

On the chromatogram of flavonoids of *R. fruticosus* L., we

found eight peaks corresponding to eight main compounds which are: gallic acid, gallocatechin, protocatechic acid, catechin, caffeic acid, rutin, ellagic acid and myricetin. Results are shown in Table 3 and Figure 1. An analysis by HPLC made in South America by Mertz et al. (2007) showed the presence of flavonol hexoside-malonates and hydroxycinnamic acids in *R. fruticosus* extracts.

A study which was realized by the same authors using HPLC with diode array reveals the presence of caffeic acid in two neighbors species which are *Rubus glaucus* Bent and *Rubus adenotrichus* Schlech, this two plants are not rich in this component, the same component was find using HPLC in a variety of *R. fruticosus* obtained from the region of Tizi Ouzou in Algeria, that shows the presence of caffeic acid in the botanical kind, *Rubus*.

Another study has revealed, using HPLC, the presence of ellagic acid and 3,4-dihydroxy-benzoic acid in the flavonoids issues from leaves of *R. fruticosus* gathered in Bulgaria. Ellagic acid was also detected in the aerial part of the studied plant. Other molecules were identified in this study and were never identified in previous studies,



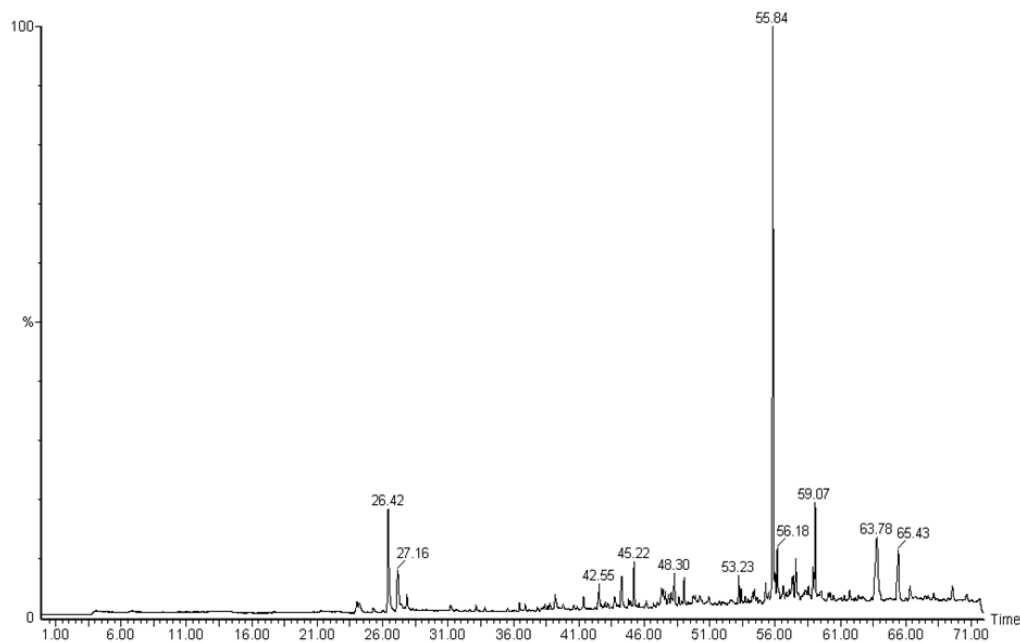


Figure 2. Chromatogram of GC-MS applied to essential oils.

Table 4. Retention time of essential oil compounds.

R <sub>t</sub>	Name of the compound	%
26.42	Limonene	13.09
27.16	Trans-3-carene-2-ol	4.21
42.55	Caryophyllene	3.13
45.22	Thymol	5.59
48.30	Veridiflorol	4.02
53.23	Epiglobulol	4.22
55.84	Lanceol	20.22
56.18	Globulol	5.15
59.07	Methyl steviol	14.12
63.78	Fenitrothion	12.85
65.43	Benzene, dodecyl	12.01
Total		98.61

this may be explained by the difference of the area of harvest (Milivojevic et al., 2011; Radovanović et al., 2013; Carlsen et al., 2003).

#### GC-MS of essential oil of *R. fruticosus*

By analyzing the essential oil of *R. fruticosus* using GC-MS, we identified 11 molecules (Figure 2). The major component is the lanceol (20.22%) (Table 4). Concerning the essential oil of *R. fruticosus*, no studies were done on it or any essential oil of any species belonging to *Rubus* family.

#### Antimicrobial activity

Table 5, the antimicrobial activity of flavonoids extract and the essential oil on reference strains are represented, both substances have an effect on the respiratory flora. The activity of both extracts was evaluated on strains isolated from patients with respiratory infections; these patients were diagnosed with respiratory infections, ORL infections or bronchitis infections.

Clinical strains chosen to evaluate antimicrobial activity showed an important resistance to conventional antibiotics, Table 6 shows the results. By assessing the antimicrobial activity of this species against some respiratory pathogens, this plant seems to be promising but no many works was done on evaluating the effect of the extract of this species against microbes. A study evaluated the activity of the ethanolic extract of *R. fruticosus* against *Helicobacter pylori* which was resistant to nalidixic acid, this extract had a MIC of 400-450 µg/ml (Abachi et al., 2013).

Another study showed that the blackberry juice inhibits the growth of *Bacillus cereus*, *Bacillus subtilis*, *Streptococcus marcescens* and *Escherichia coli* with percentages varying from 50 to 75% (Poyrazolu and Biyik, 2010).

Flan et al. (2011) found that the methanolic extract of aerial parts of *R. fruticosus* inhibits the growth of *Mycobacterium tuberculosis* with a MIC of 1 mg/ml. Another study by Riaz et al. (2011) on methanolic extracts from various parts of the plant against eight reference bacterial strains (*Salmonella typhi*, *E. coli*,

**Table 5.** Results of the antimicrobial activity against reference strains.

Strain	Reference	MIC <sub>s</sub>	
		E.O (mg/ml)	Flavonoids (mg/ml)
<i>E. coli</i>	ATCC 11229	R	R
<i>Enterobacter cloacae</i>	ATCC 13047	0.5	2.36
<i>Staphylococcus aureus</i>	ATCC 6538	R	4.72
<i>Haemophilus influenzae</i>	ATCC 10211	0.25	4.72
<i>Streptococcus pneumoniae</i>	ATCC 10015	R	4.72
<i>Pseudomonas aeruginosa</i>	ATCC 15442	0.25	R
<i>Klebsiella pneumoniae</i>	ATCC 13883	C.E	37.77
<i>Candida albicans</i>	ATCC 10231	R	37.77

R: Resistant.

**Table 6.** Activity of *R. fruticosus* extracts against respiratory pathogens.

Strains	Antibiotics									Essential oils (CIM <sub>s</sub> )	Flavonoids (MIC <sub>s</sub> ) (mg/ml)
<i>E. coli</i>	Cip	Amo	Ceph	Tic	Trim	Cefo	Cefu	Ceft			
1	R	R	R	R	R	R	R	R	R	R	C.E.
2	R	R	R	R	R	R	R	R	R	R	C.E.
<i>P. Aeruginosa</i>	Fost	Imi	Levo	Amik	Cet	Cip	/	/			
1	R	R	R	R	R	R	/	/	0.5	37.77	
2	R	R	R	R	R	R	/	/	0.5	18.88	
<i>P. fluorescens</i>	Azith	Cipr	Levo	Tic	Trim	Gent	Imi	Cef			
1	R	R	R	R	R	S	S	S	0.125	09.44	
2	S	R	R	R	S	R	R	R	0.125	18.88	
<i>B. Cepacia</i>	Amox	Imi	Gent	Cefo	Col	Cefa	/	/			
1	S	S	S	S	S	R	/	/	0.25	04.72	
2	S	S	S	S	S	S	/	/	0.125	04.72	
<i>H. Influenzae</i>	Cot	Amo	Ofi	Tet	Cefo	/	/	/			
1	R	S	S	R	S	/	/	/	0.25	02.36	
2	R	S	S	R	S	/	/	/	0.25	02.36	
<i>K. pneumoniae</i>	Amo	Cef	Gent	Amp	Ami	Col	Imi	Cot			
1	R	R	R	R	R	R	S	R	0.5	18.88	
2	R	R	R	R	R	R	R	R	0.25	18.88	
<i>A.baumannii</i>	Tic	Pop	Cef	Imi	Ofi	Cip	Col	Tic + Ac			
1	R	R	R	R	R	R	S	R	C.E	37.77	
2	R	R	R	R	R	R	S	R	R	75.54	
<i>E. cloacea</i>	Azith	Cefac	Ceft	Cefur	Eryt	Peni	Tetr	Amo			
1	R	R	R	R	R	R	R	S	0.5	37.77	
2	R	R	R	R	R	R	R	S	0.5	37.77	
<i>S. aureus</i>	Vanc	Oxa	Gent	Amp							
1	S	S	S	R	/	/	/	/	R	02.36	
2	S	S	S	R	/	/	/	/	R	02.36	
<i>S. Pneumoniae</i>	Azith	Oxa	Gent	Amp	Amo	Imi	Tetr				
1	R	R	R	R	R	R	S	R	R	18.88	
2	R	R	R	R	R	R	R	R	R	18.88	
<i>C. albicans</i>	Azith	Oxa	Gent	Amp	Amo	Imi	Tetr				
1	R	R	R	R	R	R	R	R	R	18.88	
2	R	R	R	R	R	R	R	R	R	37.77	

R: Resistant; S: sensitive.

*Streptococcus aureus*, *Micrococcus luteus*, *Proteus mirabilis*, *Bacillus subtilis*, *Citrobacteri sp.*, *Pseudomonas aeruginosa*), revealed that all the extracts inhibit the growth of bacteria with different MICs.

All these studies showed that different extracts of *R. fruticosus*, especially phenols, have a big potential to inhibit the growth of bacteria or fungus.

## Antioxidant activity

### DPPH test

Free radical scavenging was appreciated using DPPH discoloration test that revealed a high antioxidant activity of the flavonoid extract of *R. fruticosus*. This test revealed a weak scavenging of free radical activity for the essential oil, the  $EC_{50}$  was  $9.24 \pm 0.48$  mg/ml. This value is  $2.68 \pm 0.88$  mg/ml for flavonoids. In Figure 3, these results are compared with ascorbic acid as a standard.

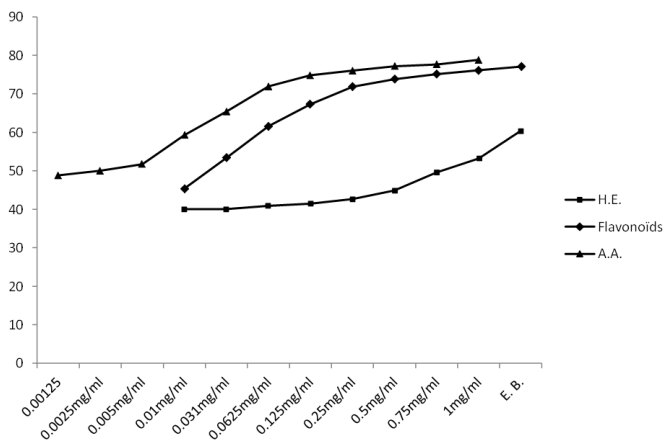


Figure 3. Antioxidant activity using the DPPH test.

The antioxidant activity of flavonoids of *R. fruticosus* is closer to the values of reference which is ascorbic acid ( $IC_{50}$  was found  $1.46 \pm 0.01$  mg/ml).

A study showed that  $IC_{50}$  varies from 15.2 to 76.5  $\mu$ g/ml for different extracts including n-hexane, dichloromethane, chloroform, ethyl acetate, methanol. Blackberries are a rich source of natural antioxidants as they contain high levels of phenols and flavonols and are therefore well reputed scavengers and inhibitors of free radicals (Ivanovic et al., 2014).

The study of both biological activities: antimicrobial and antioxidant of the extract of flavonoids and essential oil of *R. fruticosus* reveals that the essential oil has a weak biological activity while flavonoids seem to have strong biological effects.

## Conclusion

By assessing the antimicrobial and antioxidant activities of the flavonoid extract and the essential oil of Algerian *R. fruticosus* L., we can assume that this species and particularly its flavonoid extract have a remarkable inhibitory action against resistant respiratory pathogens.

More advanced studies are needed to investigate the possibility of developing formulations for pharmaceutical products from this extracts.

## Conflict of interests

The authors did not declare any conflict of interest.

**Abbreviations:** C.E, Crude extract;  $EC_{50}$ , efficient concentration 50; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MIC, minimal inhibitory concentration;  $R_t$ , retention time; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrophotometry; AMP, ampicilin; IMI, imipenem; GENT, gentamicin; AZITH, azithromicin; CIP, ciproflaxin; VAN, vancomycin; OXA, oxacilin; TET, tetracilin; ERYT, erythromicin; PENI, penicilin; AMO, amoxicilin; CEF, cefazolin; LEV, levoflaxin.

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Full Length Research Paper

# Evaluation of plant products and antagonistic microbes against grey blight (*Pestalotiopsis theae*), a devastating pathogen of tea

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The present study was carried out to evaluate the effect of different oils (lemongrass, neem, karanj, zinger, eucalyptus and patchouli oils), different plant product extracts (garlic, zinger, false ashoka and datura) and different antagonistic microorganisms (different species of *Trichoderma* spp., *Bacillus* sp. and *Pseudomonas* sp.) on grey blight of tea, *Pestalotiopsis theae*. Results reveal that eucalyptus oil and neem oil (0.05%) showed 98.1 and 94.3% inhibition of mycelial growth over the control, respectively. Although, both of them at 0.1% showed 100% inhibition for the pathogen. Similarly, plant extract garlic and datura showed 98.2 and 95.4% inhibition of mycelial growth over control. Among the different antagonistic agents, *Trichoderma viride* showed 74.3% inhibition of mycelial growth over the control. Among chemical fungicide, bavistin showed 100% inhibition over control. The various antifungal extracts showed inhibitory/fungicidal effect against grey blight of tea. These could serve as sources for development of new antifungal agent.

**Key words:** Plant products, antagonistic microorganism, grey blight of tea, mycelial growth.

## INTRODUCTION

Many farmers do not use synthetic pesticides, and some consumers will only buy organic produce. Many plant species produce substances that protect them by killing or repelling the insects that feed on them (Pelegrini et al., 2006). Plant based products has been used for many centuries among limited resource farmers in developing countries to control insect pests of both field crops and

stored produce, but their potential was initially limited and ignored (Chowdhury et al., 2003). Nowadays, scientists are exploring a lot of plant based products which are significantly used for controlling various diseases. Essential oils are steam volatile, aromatic oils different from fatty oils and the oils are obtained by enzymatic action (Nariman et al., 2009). Recently, essential oils are used

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for controlling various pests. Essential oils are natural anti-depressants, antibacterial fighters and immune strengtheners. It is a major group of agro-based industry products. Insect-pests like plants, can be infected by disease-causing organisms such as bacteria, viruses and fungi. Under some conditions, these naturally occurring organisms multiply quickly to cause disease outbreaks or epizootics that can decimate an insect population. These are called antagonistic microorganisms. Many microbial antagonists have been reported to possess antagonistic activities against plant fungal pathogens (Dabur et al., 2004).

Tea is an economically important crop which is cultivated extensively throughout north-east India. Several fungal pathogens produce foliar diseases of tea. Tea leaves are harvested from the young twigs but the lower leaves are not harvested but they are important for maintenance of the tea plants (Almada-Rui et al., 2003). One of the important leaf disease caused by *Pestalotiopsis theae* frequently attack tea plants in the sub-Himalayan West Bengal. Till date, the disease is controlled by chemical fungicides in most of the tea estates situated in the region. Use of chemical fungicides is becoming unpopular due to all round awareness of their hazardous effect on the environment (Bansod and Rai, 2008; Fukai et al., 2003). The application of broad-spectrum chemical fungicides is the common practice in most of the horticultural crops for controlling fungal diseases. The fungicides are extremely hazardous to our health and environment (Rocha et al., 2004). Therefore, it is essential to adopt eco-friendly methods to control fungal diseases of our vegetable crops. Numerous plant extraction, essential oil and antagonistic possessing potential pest-controlling properties under controlled condition, that is, in laboratory, but the step from the laboratory to the field eliminates many contenders, even when judged only on their efficacy against target pests under realistic field conditions. Keeping the export value of organic tea in foreign country, that is, chemical pesticide free fresh organic tea, *in vitro* study was carried to evaluate different plant products against grey blight (*P. theae*), a pathogen of tea plant. It is likely that the results will broaden the scientific base upon which total control of the fungal diseases of tea may be established through eco-friendly disease management programmes.

## MATERIALS AND METHODS

### Pathogen

Infected tea plants (variety TV-9) were collected from tea nurseries of Kharibari, Siliguri, West Bengal, India and used in this experiment. Fungal pathogens (*P. theae*) were isolated from the infected tea plant leaves and grown in potato dextrose agar medium and maintained in the laboratory. The pathogens were subjected to Koch's postulates for verification of the diseases.

Thereafter, they were incubated at 4°C. After seven days of incubation, the hyphal tip of the fungus radiating from the infected

tissue was transferred onto PDA slants. Freshly prepared sterile PDA slants were used for the maintenance of the fungal cultures by sub-culturing periodically. Pathogens grown on sterile PDA media were stored in two different conditions, viz. at low temperature in refrigerator (at 4°C) and in incubator at 27±1°C. At the interval of one week, subculture was done taking sample from incubator at 27±1°C for preparation of inoculums for different experiments. To avoid loss of virulence, fresh isolations were made as and when required and the pathogenicity of the isolates on tea plant leaves was ascertained.

### Plant oils

Different plant oil: lemongrass oil (0.05 and 0.1%), neem oil (0.05 and 0.1%), karanja oil (3%), zinger oil (1%) and eucalyptus oil (0.01 and 0.1%) were obtained from local market. The oil cakes were soaked in sterile distilled water individually at 2 g/ml and kept overnight for extraction by microwave assisted hydrodistillation as described by Rodriguez et al. (2012).

### Plant extract

To obtain alcohol extract from fresh plant parts, fresh plant parts were washed thoroughly with sterile distilled water and surface water was soaked by blotting paper at room temperature. The materials (3 g) were ground in a 'mortar and pestle' with 6 ml 50% ethanol with autoclaved water to make 0.5 g/ml concentration. The extracts were filtered through double-layered muslin cloth and centrifuged at 10,000 g for 30 min. The supernatant of alcohol extracts were collected in plastic vials and all extracts were stored at 4°C until used for bioassay.

### Bioagents

Antagonistic microorganisms viz., *Trichoderma viride*, *Trichoderma virens*, *Trichoderma harzianum*, *Trichoderma longibrachiatum*, *Trichoderma reesi*, *Bacillus subtilis*, *Pseudomonas fluorescens* isolate 1 and *P. fluorescens* isolate 2 were purchased from New Agriculture, Kolkata, India. These were tested against the growth of *P. theae in vitro* by dual culture technique.

### Efficacy of different oils against *P. theae*

Experiment (pre standardized) was carried out with different concentration for different plant oils. Then the concentration of plant oil for testing the efficacy was finalized. After emulsifying with teepol at 1 ml/L, appropriate concentration of plant oil (eucalyptus oil 0.05 and 0.1%, Neem oil 0.05 and 0.1%, Karanj oil 0.1%, lemongrass oil 1%, zinger oil 1%, Patchouli oil 1%) mixed with sterilized potato dextrose agar medium (PDA). Then they were thoroughly mixed just before plating to get specified concentration of the plant oils. Then 20 ml of this mixture was poured into a sterilized petri dish (10 cm diameter) in three replications to allow solidification. Culture disc of the pathogen *P. theae* having 6 mm in size was taken and placed onto the centre of the medium. The plates were incubated at 27±1°C. When the control plate showed full growth, then radial growth of the colony in each plate was measured.

### *In vivo* antifungal activity of plant extracts

Antifungal activity of ethanolic extracts (1.0 mg/ml) of garlic (*Allium*

**Table 1.** Effect of different plant oils on disease occurrence of grey-blight (*Pestalotiopsis theae*) in tea cut shoot.

Plant oil	Mycelial growth (cm)	Per cent inhibition over control
Eucalyptus oil (0.05%)	0.03	98.1
Eucalyptus oil (0.1%)	0.00	100
Neem oil (0.05%)	0.08	94.3
Neem oil (0.1%)	0.00	100
Karanj oil (1%)	1.02	85.4
Lemongrass oil (1%)	1.31	78.4
Zinger oil (1%)	1.71	71.3
Patchouli oil (1%)	2.13	62.7
control	8.87	0.00
CD ( $P=0.05$ )	1.15	

*sativum*) bulbs, datura (*D. stramonium*) and false ashoka (*Polyalthia longifolia*) leaves and zinger (*Zingiber officinale*) rhizome was tested *in vivo* by the cut shoot technique. Tea twigs (10-12 cm long) were collected from plants of susceptible varieties grown in the experimental garden and placed into the holes of a Styrofoam board and floated on Hoagland and Knop's solution in a glass chamber. The extracts were sprayed to leaves of tea-twig until runoff, 24 h prior to inoculation with the pathogens. In all cases, 0.05% Tween-20 was added to all spraying solutions as wetting agent. Control plants were sprayed with distilled water. Disease index was computed on the basis of visual observations of lesions following the technique of Sinha and Das (1972).

#### Efficacy of antagonistic bacterial microbes against *P. theae*

To carry out this experiment, a 6 mm actively growing PDA culture disc of the pathogen was placed on a PDA petri dish at one side, 1.5 cm away from the edge of the plate. This was then incubated at  $27\pm 1^\circ\text{C}$ . After 48 h, actively growing cultures of the respective test bacteria (*Bacillus* and *Pseudomonas*) were separately streaked onto the medium at the opposite side of the plate, 1.5 cm away from the edge. The plates were incubated at  $27\pm 1^\circ\text{C}$ . Three replications were maintained for each antagonistic bacteria. The radial growth of the pathogen was measured four days after inoculation. The results were expressed as per cent growth inhibition over control. The plates inoculated with the pathogen alone served as control.

#### Efficacy of antagonistic fungal microbes against *P. theae*

Culture disc having 6 mm size of actively growing *P. theae* was placed onto sterilized petri dish containing previously plated and solidified Czapek's Dox medium which are at 1.5 cm away from the edge of the plate. Another 8 mm fresh culture disc of antagonistic organism was placed opposite to *P. theae* disc. For each test three replications were maintained. The plates were incubated at  $27\pm 1^\circ\text{C}$ . After full growth of the control sample, the radial growth of pathogen in each plate was measured. Czapek's Dox medium which is inoculated with pathogen alone served as control. The results were expressed as % inhibition of the mycelial growth of pathogen over control.

$$I = 100 (C-T)/C$$

Where, I = percent inhibition over control; C = growth of pathogen in control; T = growth of pathogen in treatment.

#### Evaluation of the fungicides against *P. theae*

In 100 ml Erlenmeyer flask containing 20 ml sterilized and melted potato dextrose agar medium (PDA), 0.2% of each fungicide formulation was weighed and added. Then they were thoroughly mixed by gentle swirling, poured into Petri dish and allowed to solidify. A 6 mm actively growing mycelia disc of the pathogen was placed onto the medium. The plates were incubated at  $27\pm 1^\circ\text{C}$ . Three replications were maintained for each test. The potato dextrose agar (PDA) medium without incorporating the fungicides and inoculated with the pathogen served as control. When the control plate showed full growth, the radial growth of pathogen in each plate was measured. The data obtained from this experiment were subjected to statistical analysis using SAS 9.3 software.

## RESULTS AND DISCUSSION

#### *In vitro* assay of plant oils against *P. theae*

Among the six different plant oils tested against *P. theae*, eucalyptus and neem oil at 0.05 and 0.1 resulted 98.1 and 94.3% inhibition of mycelia growth of the pathogen than control followed by karanj, lemongrass, zinger and patchouli oil produced 85.4, 78.4, 71.3 and 62.7% inhibition of mycelia growth (Table 1). Although, eucalyptus and neem oil at 0.1% causes 100% inhibition over control. Use of different plant oils, lemongrass, neem, karanj, zinger, eucalyptus and patchouli oil for the management of *P. theae* could restrict the growth of fungus significantly. Similar type of results has been showed by Jadeja (2003), Mdee et al. (2009) and Nariman et al. (2009).

#### Effect of plant extract against *P. theae*

For studying the effect of plant extracts *in vivo*, susceptible

**Table 2.** Effect of plant extract on disease occurrence of grey-blight (*P. theae*) in tea cut shoot.

Plant species tested	Mean disease index/shoot			
	24 h	48 h	72 h	96 h
Garlic ( <i>Allium sativum</i> )	0.00	0.04	0.06	0.11
Zinger ( <i>Zingiber officinalis</i> )	0.00	0.05	0.08	0.15
False ashoka ( <i>Polyalthia longifolia</i> )	0.00	0.03	0.06	0.14
Datura ( <i>Datura stramonium</i> )	0.00	0.04	0.08	0.12
Control	1.12	1.43	2.83	2.90

**Table 3.** Effect of antagonistic microbes on the growth of *P. theae*.

Antagonistic organisms	Mycelial growth (cm)	Per cent inhibition over control
<i>Trichoderma viride</i>	2.15	72.4
<i>T. Reesi</i>	2.79	65.2
<i>T. Virens</i>	3.14	55.4
<i>T. longibrachiatum</i>	3.27	50.7
<i>Bacillus subtilis</i>	2.91	59.7
<i>Pseudomonas fluorescens</i> isolate 1	6.78	27.8
<i>P. fluorescens</i> isolate 2	5.78	35.4
Control		0.00
CD ( $P=0.05$ )	0.53	

plant tea was treated with selected plant extracts. Following the treatment, the plant was subjected to challenge inoculation pathogens *P. theae*. Garlic and datura (1.0 mg/ml in ethanol supplemented with 0.05% Tween-20) reduced the occurrence of diseases caused by *P. theae* in the susceptible inoculated plants in comparison to control followed by false ashoka and zinger. Mean disease index was lowest in garlic (0.11) followed by 0.12, 0.14 and 0.15 in datura, false ashoka and zinger, respectively (Table 2). All these plant extracts are bio-products which may be used for field application. Similar type of results has earlier been shown by Jadeja (2003), Baka (2010) and Hadizadeh et al. (2009).

#### ***In vitro* assay of bio-control agents against *P. theae***

Among the bio-control agents *T. viride* and *T. reesi* showed significantly highest mycelia inhibition of *P. theae*. The mycelia inhibitions in the above plates were 72.4 and 65.2% respectively, over control. The bacterial antagonist *B. subtilis* was the next best antagonists in inhibiting 59.7% growth of *P. theae*. Isolate *T. virens* and *T. longibrachiatum* were the other two antagonists, which inhibited the pathogen growth to 55.4 and 50.7%, respectively. The antagonist *P. fluorescens* isolates 1 and 2 were identified as poor inhibitor against *P. theae* with the growth inhibition of only 27.8 and 35.4%, respectively over control (Table 3). *T. viride* was most effective in

controlling *P. theae* in tea under *in vitro* condition. Our study on bio control agents and their application will broaden the knowledge base on which a total control of these diseases may be established. Similar type of results has been shown by Sangeetha et al. (2011) against *Colletotrichum capsici* causing fruit rot of chilli and also by Raghavendra et al. (2009).

#### ***In vitro* assay of fungicides against *P. theae***

Among all the treatments, bavistin recorded absolute inhibition of mycelial growth of the pathogen followed by captaf that produced 82.4%, nystatin resulted in 72.4%, ziram resulted in 65.7%, copper oxy chloride in 55.7%, thiram in 41.2% and mancozeb 35.7%, respectively. Mancozeb showed the maximum mycelial growth of 6.82 cm and least percent inhibition over control (35.7%). Bavistin showed 100% inhibition of the pathogen in poison food technique. Two-three spray of bavistin recorded best control of *P. theae* (Table 4). Thus, bavistin could be used as a chemical agent for controlling the disease.

#### **Conclusions**

The result of this study suggests that locally available



**Table 4.** *In vitro* assay of chemical fungicides (0.2%) against *P. theae*.

Fungicide	Mycelial growth (cm)	Per cent inhibition over control
Bavistin	0.00	100
Captaf	1.78	82.4
Nystatin	2.14	72.4
Ziram	3.02	65.7
Copper oxy chloride	4.51	55.7
Thiram	5.73	41.2
Mancozeb	6.82	35.7
Control	8.87	0.00
CD ( $P=0.05$ )	0.62	

plant products extract and antagonistic microbes may play a great role in controlling grey blight of tea disease. This may encourage the farmers to produce organic tea to generate more revenue.

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Full Length Research Paper

# Effect of the addition of water-soluble soybean extract and probiotic culture on chemical characteristics and folate concentration in yogurts produced with goat's milk

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Four yogurts from goat's milk were elaborated and water-soluble soybean extract (WSSE) in the supplementation of 14.8 g/L and *Bifidobacterium lactis* probiotic culture at 2% were added during processing. Chemical aspects and folate concentration were evaluated during the preparation for 29 days at 4°C. Regarding protein and fat analysis, treatments with added WSSE presented higher values; the yogurt with added probiotics presented lower values. Regarding the color, the L\* and a\* parameters showed no significant differences among the yogurt treatments, the b\* parameter values were higher for treatments with added water-soluble soybean extract. The determination of minerals: calcium, iron and potassium were higher for yogurts with added soybean extract. For the sodium content, the values found in yogurts were similar (43.52; 39.00; 40.85 and 47.89 mg/100 g of product). The determination of folates in yogurt presented values of 67 mcg/100 g for the goat's milk yogurt, 135 mcg/100 g for yogurt with added WSSE, 180 mcg/100 g for the yogurt with added *B. lactis* probiotic culture and 195 mcg/100 g for the treatment with added WSSE and probiotic culture. From these results, it was verified that the yogurts presented as viable in terms of their chemical aspects during the storage time of 29 days.

**Key words:** Yogurt, water-soluble soybean extract, lactic acid bacteria, *Bifidobacterium*.

## INTRODUCTION

Yogurt is an extremely popular fermented dairy product produced by the fermentation of the milk lactic acid by the addition of a starter culture containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. It is a very versatile product that suits all tastes and occasions (Isleten and Karagul-Yuceer, 2006). Yogurts have been reformulated to include living strains of *L.*

*acidophilus* and species of *Bifidobacterium* (Shah, 2000; Maestri et al., 2014). These probiotic cultures are defined as dietary supplements, which benefit those who consume them by maintaining and/or improving the intestinal microflora balance. Probiotics have been credited with the reduction in lactose intolerance, control of intestinal infection, a reduction in the propensity for some carcinomas,

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flavor and improved nutritional quality of foods containing them, and the influence on the technological properties of milk products (Denipote et al., 2010). According to the Identity and Quality Standards (PIQ) of fermented milks, in the yogurt viable bifidobacteria count there should be at least  $10^6$  CFU/mL in the final product, until the expiration date (Brasil, 2000).

In relation to other types of milk, goat's milk presents advantages such as smaller sized fat globules, high digestibility (Frazier, 1995), essential amino acid balance which equals or exceeds the World Health Organization recommendations, high calcium, selenium and phosphate content and being rich in vitamins A and B. It is deficient in folic acid; however, the nutritional deficiency of goat's milk can be improved by the lactic fermentation process (Rao et al., 1984). According to Hugenholtz (2008), many lactic acid bacteria seem to produce some vitamins, from which the fermented product is enriched as a result of bacterial production. Fermented milk products are reported to contain high amounts of folates as a result of the additional production of this vitamin via bacteria. Thus, one can carry out natural fortification of dairy products from the choice of viable starter cultures (Bernaud and Rodrigues, 2013).

Despite the numerous benefits of goat's milk, currently there is a widespread rejection of this product leading to its low consumption (Santos, 2011). For Oliveira (2009), among the viable alternatives to stimulate the consumption of this type of milk is its use in the elaboration of milk drinks, yogurts and cheeses. The preparation of these goat's milk-based products, may present significant changes in their rheological properties, such as low consistency and a tendency towards draining, because the raw material used has a slight reduction in casein content, low proportion or absence of  $\alpha$ -s1 casein and high degree of dispersion of the casein micelle, and in addition, the goat's milk curd has semiliquid characteristics (Martin-Diana et al., 2003). Therefore, to obtain satisfactory fermented goat's milk products, stabilizer addition is recommended (Lorenzen et al., 2002).

The use of additives in yogurt, to improve consistency and reduce syneresis, is widely exploited in the industry. There are a number of stabilizers with specific properties available on the market, among these, soybean derivatives such as water-soluble soybean extract is of great importance in the preparation of food products, because they improve the nutritional value of product and affect the formation of the gel structure yogurt (Silva et al., 2012).

The chemical composition of soybeans can vary with the climate, soil type, geographic location, varieties and agronomic practices, among other factors, and the derivatives of soy products, such as soy extract, also undergo changes in their mineral content. Despite this susceptibility to change, soy and its derivatives are important sources of minerals such as potassium (K), iron

**Table 1.** Identification of yogurts according to the addition of water-soluble soybean extract (WSSE) and probiotic culture.

Yogurt	Added WSSE (20%)*	Added probiotic culture (2%)
A	No	No
B	Yes	No
C	No	Yes
D	Yes	Yes

\*WSSE addition adjusted to the milk protein content at 20% concentration, resulting in the supplementation of 14.8 g/L WSSE.

(Fe) and calcium (Ca), highlighting the very high K content (Alejandro et al., 2008).

In this context, the present study was conducted to evaluate the chemical characteristics of color and the level of calcium, iron, sodium, potassium and folates in goat's milk-based yogurts with added water-soluble soybean extract (WSSE) and probiotic culture during refrigerated storage.

## MATERIALS AND METHODS

### Milk physical and chemical analysis

The milk used in the experiment was from a goat herd through the milking of Saanen females, reared in a semi-intensive system, under adequate hygienic condition. After milking, the milk was immediately cooled to 5°C in an expansion tank, transferred to previously sanitized polypropylene drums and transported to the dairy plant where the processing was carried out. Milk and yogurt sampling and analysis were held during the months of February and April.

The physico-chemical analyzes were conducted using milk samples, in triplicate, to ascertain its quality. These analyzes consisted of pH determination by direct potentiometry in a digital pH meter (Instituto Adolfo Lutz, 1985), titratable acidity measurements (potentiometric titration with NaOH 0.1 molL<sup>-1</sup> (Brasil, 2006), density, fat percentage, total soluble solids (Brasil, 2006), defatted solids were determined by subtracting the value of the fat from the value of total solids and crude protein percentage (AOAC, 1995) using the 6.38 factor for the calculation.

### Elaboration of yogurt

The study included three elaborations of yogurts during the months of November to February. Four liters of goat's milk were used for the production of the four yogurt treatments in each production. The methodology used in this study for the development of the yogurt was based on that described by Tamime and Robinson (1991). The yogurts were prepared and identified by letters according to their processing particularities (WSSE addition adjusted to the milk protein content at 20% concentration, resulting in the supplementation of 14.8 g/L WSSE and probiotic *Bifidobacterium lactis* at 2%), as expressed in Table 1.

After the milk quality analysis, the WSSE provided by Olivebra<sup>®</sup>, was added to Treatments B and D. Subsequently, all treatments were pasteurized at 80°C for 30 min and cooled to 43°C. The different combinations of *Streptococcus thermophilus* bacterial cultures and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Grabolab

Brazil<sup>®</sup> at 0.1% and lyophilized *B. lactis* at 2% (Sacco Brazil<sup>®</sup>) were inoculated individually. The fermentation end point was determined when the yogurt reached a pH of 4.6 which occurred about 5 h after incubation. After this procedure, the yogurts were removed from the oven to reach the pH 4.5 and cooled to 15°C for addition of strawberry pulp (5%) and stored at 4°C.

The analyzes for determination of color and the minerals calcium, iron, sodium and potassium were conducted on the 1st, 8th, 15th, 22th and 29th day of the yogurt treatment post-fabrication, the folate determination analysis was performed on the 15th day, post-fabrication. These deadlines were set because the shelf life of yogurt should be around 30 days, during which the product must maintain its own characteristics, provided that it is properly refrigerated (Vedamuthu, 1991).

### Chemical analysis of yogurt

Post-acidification analysis, crude protein, ether extract and the fixed mineral residue (ash) of the yogurt treatments were performed in triplicate. The post-acidification was determined by measuring the pH using Quimis<sup>®</sup> potentiometer model Q-400 (Brazil). The protein fraction was obtained by the Kjeldahl method described by AOAC (1995); the ether extract, by AOAC (1995) and the fixed mineral residue determined by incinerating the sample at a temperature of 550°C (AOAC, 1995).

### Determination of color

The color was determined by the CIELAB system (International Commission on Illumination) in Minolta<sup>®</sup> CR 310 equipment (illuminant C or D65 and 10° angle) through the color parameters: L\* (lightness), a\* and b\* (chromaticity coordinates) measured in the equipment itself. Yogurt samples were placed in Petri dishes and the analysis was performed in triplicate.

As for the chromaticity coordinates, +a\* is in the red direction, -a\* is in the green direction, +b\* is in the yellow direction and -b\* is in the blue direction. L\* measures lightness and varies from 100 (one hundred) for perfectly white surfaces to 0 (zero) for black. The center is achromatic, as the values of a\* and b\* values increase and the point moves away from the center, the color saturation increases (Minolta, 1994).

### Mineral analysis

Minerals (calcium, iron, sodium and potassium) were determined by flame atomic absorption spectrophotometry in a Varian<sup>®</sup> Thectron AA5 spectrophotometer. 0.5 g of yogurt samples were weighed on an analytical scale and subjected to digestion with 6 mL of nitroperchloric solution for about 2 h at 140°C. Sample preparation procedures and mineral quantification were performed according to Malavolta et al. (1997). The results were expressed as a percentage.

### Determination of folates

The determination of folates in the yogurt was measured after extraction, deconjugation of polyglutamates using a conjugase ( $\gamma$ -glutamyl hydrolase) and subsequent quantification by high performance liquid chromatography (HPLC) by the method validated by Romero and Camargo (2007).

### Statistical analysis

The experiment was conducted in completely randomized design in

**Table 2.** Milk physical and chemical analysis for the goat's milk.

Physical chemical parameters	Mean (SD)**	IN n.37/2000*
Acidity (g lactic acid/100 ml)	0.17 (0.06)	0.13 a 0.18
Density (15/15°C g/mL)	1.030	1.028 – 1.034
Fat (g/100 g)	3.1 (0.4)	3.0
pH	6.68 (0.14)	-
Protein (g/100g)	3.2 (0.20)	Minimum 2.8
Defatted solids (g/100 g)	8.4 (0.12)	Minimum 8.2
Total solids (g/100 g)	11.5 (0.08)	-

\*(Brasil, 2000); \*\* SD- standard deviation.

a 2 x 2 x 5 factorial with 2 soybean extract concentrations, addition of probiotic culture in 2 treatments and 5 storage times, with three repetitions. The treatments were evaluated by analysis of variance, followed by Scott-Knott test at 5% significance level, using the R software.

## RESULTS AND DISCUSSION

### Milk physical and chemical analysis

The results of the physicochemical analysis of the goat's milk used in the yogurt treatments are shown in Table 2. The average acidity value, fat, protein, density, total solids and defatted solids of the goat's milk were within the standards of the current legislation. According to the results, it was observed that the milk used in the yogurt manufacture meets the standards required by Instruction n.51 (Brasil, 2000), characterizing it as a raw material of good physicochemical quality, which meets the legal standards required for manufacturing yogurts.

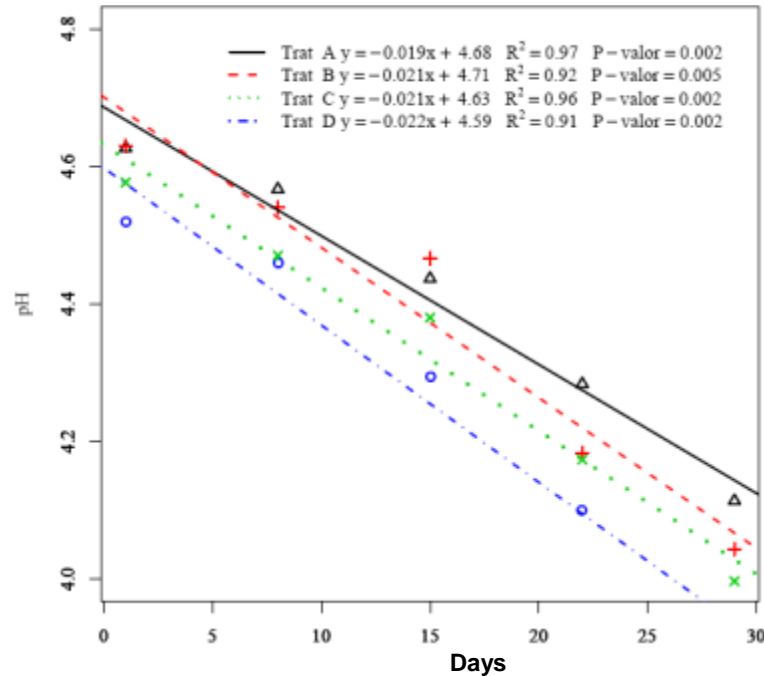
These results were similar to those found by Lora et al. (2006). The results found for pH were similar to the values obtained by research done in the state of São Paulo by Gomes et al. (2004) and Richards et al. (2001) evaluated the pasteurized whole goat's milk, and found average total solid values from 12.08 to 12.23%.

### Elaboration of yogurt

The preparation of yogurt occurred after fermentation time in an oven at 42°C for five hours, there was no change in the time between the four treatments. From a practical point of view, the fermentation time registered in this study did not exceed the normal time observed in traditional manufacturing processes, which does not preclude the use of WSSE protein.

### Chemical analysis of yogurt during storage

The results of the pH of the yogurt treatments showed



**Figure 1.** Regression model for the pH as a function of storage time at 4°C in yogurt.

variation (4.6 to 3.8) during storage at 4°C. Treatment A had the highest mean pH (4.45), Treatment B, had a mean pH of 4.38, Treatment C 4.30 and Treatment D showed an average pH of 4.22.

In the evaluation of yogurt, it was noted that there was a significant influence of the treatment time and the pH value according to the graph in Figure 1.

Results similar to the present survey were found by Assumpção (2008) with the production of cow's milk yogurt with added soybean soluble extract. According to Bortolozo and Quadros (2007), the ideal pH for fermented milks is close to 4.5, since lower values can lead to rejection by consumers and promote clot contraction due to hydration of proteins, causing draining.

In relation to the protein determinations, the yogurt averaged 2.41%, Treatment B averaged 3.2%, Treatment C 2.34% and Treatment D averaged 3.02%. It was observed that the yogurts encoded by the letters A and C had similar protein contents, showing that the addition of the probiotic *B. lactis* caused little proteolysis in these products. In goat's milk yogurt encoded by the letters B and D, the proteins values found were higher than that in the other treatments because of the addition of water-soluble extract during the manufacture.

Lee et al. (1990) evaluated the protein content of soy yogurt supplemented with whey protein concentrate or defatted milk powder. The product supplemented with protein concentrate showed 8.12% protein, while that supplemented with defatted milk powder had 7.28%, both of which had higher protein concentration than the yogurt

developed in our work. The protein content of frozen yogurt for goat's milk supplemented with probiotics evaluated by Alves et al. (2009) showed results similar to those of the present study ( $3.0 \pm 0.3\%$ ), being above the minimum recommended for the product (2.5%).

For the fat content, the average content of Yogurt A was determined at 2.44%, Yogurt B 3.86%, Treatment C 2.35% and Yogurt D 3.63%. The yogurts added WSSE showed higher average fat values of higher fat than the other treatments.

For Thomopoulos et al. (1993), milk fat favorably affects the quality of yogurt, fat stabilizes the protein contraction of the gel, prevents the separation of whey in the final product and affects the sensory perception of the product, which has more soft and creamy texture.

The average values of fixed mineral residue of yogurt with added WSSE were higher than in the other treatments, however, they showed no significant difference among themselves ( $p > 0.05$ ).

### Color determination

As can be seen in Table 3, the values of the color determination were not influenced by the addition of the water-soluble soybean extract or the addition of the *B. lactis* probiotic.

Regarding the determination of yogurts color, average values of L\* and the chromaticity coordinates a\* and b\* were not affected by storage time. The mean values of

**Table 3.** Average color values of samples of goat's milk yogurt with added water-soluble soybean extract and probiotic culture.

Yogurt	L*	a*	b*
A	71.13 <sup>a</sup>	11.16 <sup>b</sup>	0.59 <sup>c</sup>
B	73.22 <sup>a</sup>	11.74 <sup>b</sup>	1.76 <sup>d</sup>
C	72.96 <sup>a</sup>	10.85 <sup>b</sup>	0.68 <sup>c</sup>
D	72.09 <sup>a</sup>	11.38 <sup>b</sup>	1.91 <sup>d</sup>

L\* = lightness, +a\* = red, -a\* = green, +b\* = orange, -b\* = blue. Means followed by the same letter do not differ statistically among themselves, by Scott-Knott test average, to 5% probability. Yogurts: A (without addition of WSSE and without addition of probiotic culture), B (with addition of WSSE and without addition of probiotic culture), C (without addition of WSSE and with addition of probiotic culture), D (with addition of WSSE and with added probiotic culture).

**Table 4.** Mean and standard deviation (SD) of calcium, iron, sodium and potassium in samples of goat's milk yogurt with added water-soluble soybean extract and probiotic culture.

Yogurt	Calcium (mg/100 g)	Iron (mg/100 g)	Sodium (mg/100 g)	Potassium (mg/100 g)
	Mean (SD)*	Mean (SD)*	Mean (SD)*	Mean (SD)*
A	74.0 (1.4) <sup>a</sup>	1.0 (0.3) <sup>a</sup>	43.5 (0.6) <sup>a</sup>	160.0 (2.3) <sup>a</sup>
B	116.0 (1.1) <sup>b</sup>	1.9 (0.2) <sup>b</sup>	39.0 (0.2) <sup>a</sup>	166.0 (1.5) <sup>a</sup>
C	62.0 (0.7) <sup>c</sup>	0.9 (0.2) <sup>a</sup>	40.8 (1.1) <sup>a</sup>	132.0 (0.9) <sup>b</sup>
D	86.0 (0.9) <sup>d</sup>	1.3 (0.1) <sup>a</sup>	47.9 (0.8) <sup>a</sup>	190.0 (1.3) <sup>c</sup>

\*SD– standard deviation. Means followed by the same letter do not differ statistically among themselves, by Scott-Knott test average, to 5% probability. Yogurts: A (without addition of WSSE and without addition of probiotic culture), B (with addition of WSSE and without addition of probiotic culture), C (without addition of WSSE and with addition of probiotic culture), D (with addition of WSSE and with added probiotic culture).

the brightness parameter and coordinate a\* showed no significant difference between the four products analyzed ( $p > 0.05$ ). Only the parameter b\* presented higher average values for the treatments with added water-soluble soybean extract, being statistically significant. Research carried out by Moraes (2004), when evaluating traditional brands of strawberry flavor yogurt, found results similar to the present work. The positive values found in the colorimetric analysis of this parameter in yogurts indicate that there was a tendency towards yellow. This direction is caused by the addition of WSSE (Lambrecht et al., 1996). The yellowing of soybeans can vary depending on the cultivar used (hilum color, seed coat color) (Bhardwaj et al., 1999). A similar result was obtained by Ciabotti et al. (2009), on evaluating the color of soymilk and whey based products.

### Mineral composition

The concentration of the minerals in the formulations analyzed showed that the addition of soybean extract increased the calcium, potassium and iron in the product, confirming the data described above. Research by Garcia et al. (2006) to determine the mineral content in goat's

milk and dairy products derived from goat's milk produced results similar to those in the present research for the yogurt without soybean extract addition.

Table 4 shows the results of the analysis of minerals found in the yogurts in this study. In this study, the supplementation with water-soluble soybean extract in goat's milk yogurt provided an increase of calcium content (116.0 mg/100 g), however, in the yogurt in which probiotic culture was added, there was a reduction in this value; this fact can be explained by the consumption of nutrients resulting from the metabolism of these microorganisms. It is noteworthy that the probiotics, besides increasing protein and fat digestibility and reducing lactose content, also act by increasing the absorption of some minerals, especially calcium and iron (Gomes and Malcata, 2002).

Goat's milk has higher calcium and potassium levels, which may interfere with the technological characteristics of the product and its derivatives (Haenlein, 2001). However, due to the increase in temperature, pH and inoculation with microorganisms, one can see a reduction in the calcium (Gomes and Penna, 2009) and potassium (Miguel et al., 2010) concentration during the fermentation process. The four yogurt treatments showed statistically

**Table 5.** Folate content of the samples of goat's milk yogurt with added water-soluble soybean extract and probiotic culture.

Yogurts	Folate (mcg/100 g)
A	67.0 <sup>a</sup>
B	135.0 <sup>b</sup>
C	180.0 <sup>c</sup>
D	195.0 <sup>d</sup>

Means followed by the same letter do not differ statistically among themselves, by Scott-Knott test average, to 5% probability. Yogurts: A (without addition of WSSE and without addition of probiotic culture), B (with addition of WSSE and without addition of probiotic culture), C (without addition of WSSE and with addition of probiotic culture), D (with addition of WSSE and with added probiotic culture).

different average calcium values, showing that the addition of soy extract and bifidobacteria, during fermentation, change the final product.

There was an increase in the iron content in the yogurt supplemented with soybean extract and also the reduction of this mineral in yogurts with added probiotic culture. The iron concentration for samples of yogurts with defatted soybean extract, found by Miguel et al. (2010) were higher than in the present work, probably due to the type of soy extract used for the preparation of the products. This result shows that the iron concentration may vary in accordance with the soybean extract used as a thickener. With respect to sodium, the variation among the yogurt treatments was low, there were no statistical differences between the products. Fuchs et al. (2005), when determining the sodium concentration in yogurt with added soy extract and prebiotics, found values lower than those obtained in the present study. It is noteworthy that goat's milk has lower sodium concentrations than those found in cow's milk (Padua, 2013).

### Determination of folates

The determination of folates was conducted in triplicate, on the 15th day after the manufacture of the yogurts. The unfeasibility of folate detection throughout the product shelf life is due to the cost of conducting this procedure. The folate analysis results of the goat's milk yogurt with added water-soluble soybean extract and probiotic culture can be seen in Table 5.

There was a significant difference in the folate concentration values in the analyzed yogurts. All treatments with added WSSE and/or bifidobacteria had higher folate values as compared to the control yogurt. This behavior can be explained by the fact that the WSSE had contributed to the increase in the folate content of the products and also the synthesis of this vitamin by the bifidobacteria.

It can be seen that the yogurt treatment encoded by the letter A had the lowest folate concentration. Research carried out by Rao et al. (1984), in order to evaluate the biosynthesis and use of folic acid in milk fermented by various lactic cultures, showed that the association of *S. thermophilus* and *L. bulgaricus* in fermented milk increased the folate concentration, however, when the author related the folate level with associations of lactic cultures, it was observed that the samples with added *L. bulgaricus* showed lower content of this vitamin. According to Lin and Young (2000), lactic cultures not only synthesize, but also use folates.

It appears also that the C and D treatments with added probiotic culture *B. lactis* had high folate levels. Lin and Young (2000) quantified and evaluated folate stability in milk fermented by lactic cultures of *S. thermophilus*, *L. acidophilus*, *B. longum* and *L. bulgaricus* stored at 4°C, and found that the highest concentration of this vitamin in the fermented milk evaluated was the milk with addition of *B. longum* (98.0 ng/mL).

Biofortification with folate as an approach holds the promise of being cost effective because products with elevated levels of this essential vitamin would provide economic benefits to food manufacturers and increased "natural" folate concentrations would be an important value-added effect, without increasing production costs (Iyer and Toma, 2011).

### Conclusions

The levels of the minerals, calcium, iron and potassium were higher for yogurts with added soybean extract. For the sodium content, the values found in yogurts were close to each other. It was also noted that there was an increase in the concentration of folates in yogurts with added probiotic culture.

### Conflict of interests

The author(s) did not declare any conflict of interest.

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*Full Length Research Paper*

## Microbiologic quality of hand creams in Pelotas, Brazil

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The use of cosmetics has been widely accepted; among them, hand creams are commonly used with the principal function of hydration. However, for the user's safety, and to ensure good-quality products, these products should follow the Good Manufacturing Practices (GMP) and a strict microbiological quality control. In this study, six samples of hand creams, two industrialized and four compounding were evaluated by microbiological methods of counting and identification of microorganisms. In the industrialized samples, there was no fungal or bacterial growth, whereas in the manipulated ones, there was a growth in 75% of the samples, but none exceeded the limits allowed by microorganisms, and they showed none pathogenic microorganisms. Through the results, it can be concluded that the origin of manufacture may influence final product quality. The results emphasize the need for a stricter control in pharmacies to prevent contamination in the production process, and to ensure safety and trustworthiness to the users of such products.

**Key words:** Microbiology, quality control, cosmetic Industry.

### INTRODUCTION

The use of cosmetics has sharply increased in recent years both in Brazil and worldwide. By analyzing the industrial market, we observed that the national industry rose between 2003 and 2008 by 3.4%, while the cosmetic sector grew by 11.5%. Today, Brazil is already considered the third largest consumer market for cosmetics, only behind the U.S. and Japan (Leonardi, 2008).

This growth is due to the fact that nowadays both women and men are increasingly seeking for products which help them build a better personal image. Beautification, correction of imperfections and presenting a good personal image are targets of a modern society.

According to the National Health Surveillance Agency (ANVISA), the Board Resolution (RDC) number 211 of July 14<sup>th</sup>, 2005, determines that the toiletries, cosmetics and perfumes can be produced from natural or synthetic substances destined for exterior use. Cosmetics are designed to clean, perfume, hydrate, change the appearance, correct body odor, protect and keep in good appearance external features of the human body (Brasil, 2005; Ribeiro, 2010).

Among the various presentations of cosmetics, the creams used for the hands are important, since manicured hands mean concern with hygiene and welfare. Hands are one of the most vulnerable parts of

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the body, being susceptible to the effects of the environment due to being permanently without protection. In addition, they are prone to dehydration, once their backs consist of a thin and delicate skin with a small amount of sebaceous glands (Ribeiro, 2010).

The hand creams may contain moisturizing, emollient, anti-irritants, lightening, exfoliating and rejuvenating or aging preventive actives. It is the purpose of the product that is responsible for the choice of its active principles. Specific active principles must be used for the adequate purposes, being hydration and smoothing, the main reasons for seeking such formulation (Ribeiro, 2010; Kumar and Tyagi, 2014).

Moreover, cosmetic products should be prepared according to sanitary regulations, since they are substances that are used in external parts of the human body. In Brazil, ANVISA is the institution responsible for the regulation, supervision and production control, ensuring that products are safe and present good quality (Brasil, 2008).

Thus, quality control should be always performed throughout the process of cosmetics preparation. This control consists of a collection of necessary and relevant assays, which should be run during the entire production process to ensure that the medicines and cosmetics in general satisfy the quality, activity, purity, safety and efficacy desired, be such products are either processed or compound. In addition, there are the good manufacturing practices (GMP), standards, which must be followed from the beginning of the process to the final product, and guarantee the safety and quality of products offered for sale (Kumar and Tyagi, 2014; Brasil, 2008; Pinto and Kaneko, 2010; Rebello, 2004; Chorilli et al., 2007; Silva and Silva, 2011).

During quality control, physical, chemical and microbiological tests are carried out on all products that are used in the formulation (Pinto and Kaneko, 2010; Rebello, 2004). The microbial quality control of cosmetics is a quantitative assay which occurs by counting the number of microorganisms, thus evaluating the state of contamination of the product. In this counting, the presence of a limited bio burden is considered as acceptable. The absence of pathogenic microorganisms should also be evidenced. The purpose of this control was to determine the number of viable microorganisms present in formulations and perform identification of pathogenic microorganisms (Leonardi, 2008; Brasil, 2005; Pinto and Kaneko, 2010; Rebello, 2004; Andrade et al., 2005).

The microbiological quality is defined by microbial standards described in official compendia and regulatory standards. In view of the above, to produce a cosmetic quality standard, the manufacturer must demonstrate its effectiveness, safety, acceptability and credibility through validated tests that should be performed by qualified professionals (Ribeiro, 2010; Rebello, 2004; Martinelli et al., 2005; Souza and Maciel, 2010).

Besides quality control, to ensure that the product continues satisfactorily, it is necessary to use suitable preservative formulations, since they aim at inhibiting microbial growth, preserving the product. It is important that consumers are directed to use the product, making its proper use and keeping it in appropriated storage conditions (Chorilli et al., 2007; Siqueira, 2005).

Due to the great importance of microbiological control, and considering that these cosmetics are used in an area of the body that may come in contact with the eyes, mouth and mucous membranes, and therefore, can transmit such undesirable microorganisms, besides leading to serious consequences to the consumer if there are values of fungi and bacteria above the allowed levels or even the presence of microorganisms considered pathogenic, the present study evaluated the microbiological quality of hand creams.

## METHODOLOGY

Six samples of hand cream with the main function of hydration were analysed. Among these, four were from compounding pharmacies, chosen randomly among pharmacies available in the city of Pelotas. And two samples were industrialized, acquired randomly in shops in the same city.

Compounding pharmacies make their cosmetics under a prescription or request from the user, while industrial pharmacies have a stock production and national/global distribution system. From both sources, was chosen the same reagents for both products, so it was possible to compare the quality of each manufacture method.

All samples were within the expiration date. The samples are identified by letters from A to F, with A, B, C and D regarding compound pharmacies, E and F of industries.

Evaluation of organoleptic characteristics was performed on all samples before testing, where color, odor and appearance were analysed. Microbiological analyses were performed by counting total viable microorganisms and subsequent identification of pathogenic microorganisms.

### Total viable count of microorganisms– plate count method

The technique of counting the total viable microorganisms was adapted according to the Brazilian Pharmacopoeia (Brasil, 2010). The adaptation was necessary because the objective of this study was to analyze the final product with its conservation proprieties.

For the dilution of samples, 10 g of each analysed cream was transferred for a volumetric flask containing 90 mL of soybean-casein broth with 0.1% sodium tetradecyl previously prepared, heated to 45°C, stirring until a homogeneous mixture was formed.

For the detection of bacteria, 5 mL of dilution of each sample was pipetted into Petri dish. After this procedure, 30 mL of soybean-casein Agar medium liquefied at 45°C was added. The agar was mixed homogeneously with the sample, mixture which was expected to solidify. The samples were incubated at 30-35°C. Observation was made daily for four days. In the samples in which bacterial growth was observed, the number of colonies was quantified using a colony counter with controlled artificial lighting.

For detecting fungi, 5 ml of dilution of each sample was pipetted into Petri plate. After, 30 ml of Sabouraud-dextrose Agar medium liquefied at 45°C was added. The agar was homogeneously mixed into the sample until it solidified. The samples were incubated at 20-

25°C. Observation was performed in a daily basis up to seven days. The number of colonies was counted in the samples which presented fungal growth using a colony counter with controlled artificial lighting.

All samples were performed in triplicate, both in the tests for bacteria and for fungi. The negative control was performed by adding 5 mL of sterile water in a Petri plate, to which was added 30 mL of soybean-casein agar liquefied at 45°C. Likewise, the negative control was performed using Sabouraud dextrose agar. In addition, we performed a control of the means employed by adding them alone on the plates. The positive controls of *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739) and *Candida albicans* (ATCC 10231) were also made.

### Identification of pathogenic microorganisms

After the counting conducted by using the artificial lighting counter, colonies with different morphological characteristics were identified. In order to permit the isolated growth of the colony, they were sown in simple broth. The tube was incubated in an oven for 24 h. After the growth, Gram staining was performed for prior identification of microorganisms.

Colonies with Gram-negative characteristics were plated on MacConkey. The ones with Gram-positive Cocci characteristics and Gram-positive bacilli were plated on blood agar. These were incubated at 30-37°C in an oven for 24 h. For identification of *S. aureus*, we used the technique of the coagulase tube. The Gram-positive bacilli were not identified because they are not described in the Brazilian Pharmacopoeia (Brasil, 2010). The identification of fungi was performed by colony morphology. In the case of colonies with characteristics of *C. albicans*, the Chromium agar test was conducted for identification.

## RESULTS AND DISCUSSION

Microbial contamination of pharmaceuticals, whether processed or compound, can be derived from various origins, either due to the complex production process, the contaminated raw material or from other sources. Due to this fact, companies should always ensure that all process steps follow GMPs, and that there is staff trained for this function, since the production process is the responsibility of manufacturers (Brasil, 2008; Pinto and Kaneko, 2010; Yamamoto et al., 2004; Bazzo et al., 2006). Besides the preparation, quality control at all stages, as well as in the final product, should be serviced by companies. This control includes both physico-chemical and microbiological tests.

The microbiological quality control of cosmetic formulations should be performed mainly because the preparations consist of vehicles and active principles that constitute an ideal environment for the growth of microorganisms, since it can find in cosmetics, a source of carbohydrates, proteins, amino acids, vitamins, organic salts, water, among others (Bazzo et al., 2006). It is extremely important to evaluate water quality, since this is the main component of cosmetics and a major source of contamination (Martinelli et al., 2005).

The importance of evaluating the water is evident in the article of Andrade et al. (2005), which analysed 59

samples of water for pharmaceutical use. It was found that 44% of the samples were in disagreement with the pharmacopoeia specifications. Among them, there were samples with contamination above the limit allowed. In a sample of deionized water, for instance *E. coli*, a pathogenic microorganism was found. This evaluation confirmed that water quality can contaminate pharmaceuticals, which points out to the necessity of having a stricter quality control of such a raw material.

The microbial contamination of the product can also be influenced by the whole process of manufacturing, raw material control, equipment and facilities sanitization, material packaging, cleaning procedure, environmental and operational conditions (Andrade et al., 2005; Bazzo et al., 2006; Associação Brasileira de Cosmetologia, 2008). Therefore, cosmetics are subject to the growth of fungi and bacteria, a fact which depends on the product composition, water availability, storage temperature, hygiene in manufacturing, among others (Chorilli et al., 2007).

This study evaluated six samples of hand creams, of which two were from industrialized sources, and four were from compound sources. Among the industrial samples tested, there was no growth of microorganisms during the days of observation. The results shown in Table 1 suggest that these industries have stricter control policies and are correctly following quality control and the Good Manufacturing Practices.

The GMPs for the toiletries, cosmetics and perfumes industry were established by the ANVISA and seek compliance with the guidelines established in the technical regulation handbook of good manufacturing practices, with the goal of protecting public health (Chorilli et al., 2007). Besides the requirement of GMPs, another factor reducing the chances of contamination is that industries buy raw materials in large quantities, which avoids the fractioning of the material, and the fact that the entire production is made in closed batches (Yamamoto et al., 2004). Therefore, there are differences in the possibility of microbial contamination in cosmetics depending on the manufacturing origin.

There are also the Good Compounding Practices (GCP), these compound products should follow a stricter standard, which goes beyond what the Good Manufacturing Practices, the GMPs, prescribe. The GCPs aim to analyze all the inputs involved in the production process, and ensure that the compound products are manufactured, transported, stored and distributed securely, reducing the chances of microbial contamination and other physical and chemical changes (Medeiros et al., 2007; Marques and Moreira, 2009). In spite of the existence of such resolution, many pharmacies have not yet deployed the microbiological quality control on products that are not sterile (Medeiros et al., 2007).

In this study, from the four samples of hand cream manufactured in compounding pharmacies that were

**Table 1.** Growth of microbial samples in compound and processed hand creams.

Sample	Bacteria(CFU/g)	Microbial growth		Microorganisms found (UFC/g)
		Fungi (UFC/g)	Microorganisms count (UFC/g)	
*A	-	1	1	Non identified
*B	21	14	35	Gram-positive Bacilli/Gram-positive Coccus
*C	-	-	-	-
*D	2	-	2	Gram-positive Bacilli
**E	-	-	-	-
**F	-	-	-	-

\*Compound samples, \*\*industrialised samples.

analysed, there was bacterial growth in two and fungal growth in one (Table 1). This may have occurred because in the compounding pharmacies, the preparation of the formulations is prescribed individually, which requires acquiring a smaller amount of raw material. This factor ends up leading to the material fractioning in the distribution company, which may create possibilities to the microbial contamination (Yamamoto et al., 2004).

It is important to highlight that raw materials often come from the distribution companies contaminated. Therefore, the quality control should start even before the production process. Besides fractioning, the procedure is individualized, which can increase the chances of contamination.

Microbial growth can also happen because pharmacies still have a less strict control than industries. This fact can as well result from contaminated water, as this is the main component of cosmetics and often ends up contaminating the products. Besides these facts, among others, there is also the training of employees, which often can contaminate the formulations accidentally or due to poor hygiene. In Table 1, we can observe the growth of microorganisms in the samples.

The microbial growth may or not cause visible modifications in formulation. Loss of stability may occur by breaking the emulsion, alteration in physicochemical characteristics, and changes in color, odor and consistency of the product. It can also undergo degradation and inactivation of active ingredients and excipients, damaging the material and causing loss of effectiveness, causing the consumer to abandon its use (Andrade et al., 2005; Yamamoto et al., 2004; Blume et al., 2007). Furthermore, contamination can lead to a high health risk, since this can occur by pathogens or saprophytes in a high number (Yamamoto et al., 2004).

During the visual inspection of products, no samples showed alterations or modifications in its organoleptic characteristics. However, in this case, there was no relation between the contaminated creams and their characteristics, which did not differentiate a contaminated

from an uncontaminated cream.

Since this contamination can occur by pathogenic microorganisms, the development of microbial growth must always be identified in order to avoid the presence of these microorganisms. According to the Brazilian Pharmacopoeia (Brasil, 2010), for pharmaceutical topical non-sterile products, the presence of the following pathogens is not accepted: *Salmonella* sp., *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *Serratia marcescens*, *Klebsiella* sp., *Pseudomonas cepacia*, *Pseudomonas maltophilia*, *Pseudomonas stutzeri* and group B *Streptococcus*. It also determines the acceptable values of non-pathogenic microorganisms and determines which pathogens should be absent in non-sterile product formulations. Cosmetics used for the area of the hands belong to the product group type I. For such products, the limit of non-pathogenic microorganisms is a maximum of  $10^3$ CFU/g and still there should not be sign of the pathogen microorganisms like *P. aeruginosa*, *E. coli* and *S. aureus* (Brasil, 2010).

The present study was performed to identify pathogens. The results found showed colonies with morphology and microscopy of Gram-positive Coccus (Table 1). However, having the coagulase technique performed, a negative result was obtained, which led to the identification of coagulase-negative *Staphylococcus*.

The results also evidenced colonies with morphology and microscopy of Gram-positive bacilli (Table 1). Nevertheless, these were not identified because they are not considered pathogenic for this type of sample according to the Brazilian Pharmacopoeia (Brasil, 2010). There was no growth among the strains of Gram-negative bacilli. To identify the fungi, the observation of colony morphology was performed. It was observed that there were filamentous structures with varying sizes and color. Thus, it was found that these microorganisms were not *C. albicans*, because according to Zaitz et al. (2010), this fungus is yeast, and is present in the culture medium as small colonies and white coloring.

The positive and negative controls behaved as expected.

No samples exceeded the limit allowed by law, and none was contaminated with pathogenic microorganisms. According to the data presented here, there was growth in 50% (3/6) of the analysed samples. However, when evaluating the manufacture origin of the products, 75% (3/4) of the compound samples were contaminated.

These results are similar to those of Chorilli et al. (2007), who analysed six samples of compound cream for the eye area. In such research, there was no product with pathogenic bacterial contamination or with the level of pathogenic microorganism above the established limits. Nevertheless, two samples were with fungi values above the permitted, suggesting that those pharmacies should follow more strictly the GMPs and GCPs to minimize these contamination values.

In another study, Blume et al. (2007) analysed five samples of pressed powder, and found that 20% of these were contaminated with microorganisms, but, in this case, contamination was above the limit set by law.

In the study of Yamamoto et al. (2004), a total of 260 samples, from raw materials, pharmaceutical products, cosmetics and herbal medicines from 25 producers were analysed. Of these, 92.3% were approved and 7.7% were rejected. Among the rejected, 5% presented microbial contamination above the established limit of both bacteria and fungi. Among the cosmetic analysed, two were contaminated with *E. coli* and *P. aeruginosa* and one with *S. aureus*, which proves that the quality control of products should be more rigorous, since such contamination can harm the health of users. For the authors, the results of this study pointed out faults in the manufacturing process, where standard operating procedures should be evaluated and preventive measures to improve product quality should be established.

Marques and Moreira (2009) conducted a similar study in which 13 samples of compound sunscreens were analysed, of which 53.84% were rejected. Among these, seven were contaminated with *S. aureus* and *P. aeruginosa*.

In addition, some samples were above the legally set limits for bacterial and fungal presence. According to the authors, this contamination was probably generated by reason of noncompliance with the good compounding practice and could be avoided or minimized through acts as washing hands, wearing gloves and clean coats and tying the hair. Another factor that may have caused the contamination may have been water or other contaminated raw materials.

Medeiros et al. (2007) analysed nine samples of compound products, including syrups and cosmetics. Among these samples, 54.5% were contaminated and/or above the specified allowed limits. This result can be derived from the lack of quality control in raw materials or the contamination which may have occurred during production.

Although, it is uncommon for a contaminated hand

cream to reach the blood stream and cause a major infection under normal circumstances, the contamination can cause minor adverse reactions, like an erythema (Dupont et al., 2013). In the study of Huf et al. (2013), 200 people from the administrative staff of the Municipal Guard of Rio de Janeiro (Brazil), were analyzed and was found that 38% of the participants had adverse reactions to cosmetics use. While Huf et al. (2013) study did not investigate the cause of the reactions, microbial contamination could be a factor.

One of the major obstacles to the cosmetic health security, is the lack of an open knowledge integration system. Each finding from the cosmetic industry is still considered "internal knowledge" preventing other industries from copying their techniques and technology. This knowledge blockage is very detrimental to public health, as it makes more difficult the standardization of the process as discussed in this study (Celadon, 2014).

Given the above, in this work, it was observed that all tested samples were acceptable according to the current legislation, although there is contamination on three samples, however, below the specified threshold. This fact highlights the need for greater rigor in the production process which has to be adopted by compounding pharmacies, since there was a growth in 75% of the analysed compound samples. With the implementation of more stringent GMPs and GCPs these contaminations could be avoided. Moreover, in pharmacies in general, the use of preservatives is smaller, since samples are produced with a shelf life of about six months, which may also justify such microbial growth.

In the industrialized samples, there was no growth registered, which implies that the origin of the industrial process is more rigorous. However, in this case, the possibility of using a larger amount of preservative is not discarded, considering that the formulations are manufactured to have a shelf life of two years on average. However, the amount of preservatives has not been evaluated in this study.

## Conclusion

Although, the present study has a low number of samples, the findings are fascinating: it can be concluded that 50% of the samples were contaminated, but all the contamination was within the group from a compound source (75% contamination in compound hand creams). However, the contamination was below the specified threshold and there was an absence of pathogens, being all considered as good quality and proper to use.

While the products analysed had an acceptable microbiological quality, the results imply that the manufacturing origin may have an influence in the quality of the final product, suggesting the necessity of a review in the GMP to ensure no difference between compound and industrial source.

## Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

## Meloidogyne incognita and Fusarium oxysporum interaction in Gerbera

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Pot culture experiments were carried out to study the interactive effect of root knot nematode, *Meloidogyne incognita* and the fungus, *Fusarium oxysporum* in Gerbera, *Gerbera jamesonii* Hook. Sequential and concomitant inoculation of the nematode and fungus was done to observe the disease severity in the plants due to the individual effect and interactive effect of nematode and fungus. It was observed from the study that the wilt disease was found to be more severe with the sequential inoculation of nematodes followed by fungus than with the fungus alone treatment. From the study, it was clear that nematodes act as a predisposer in the spread of secondary fungal pathogens.

**Key words:** Gerbera, *Meloidogyne incognita*, *Fusarium oxysporum*.

### INTRODUCTION

Gerbera (*Gerbera jamesonii* Hook) is a popular cutflower widely used as a decorative garden plant. It is the fifth most used cutflower in the world. In India, there is an increase in demand for this cutflower which is fetching one of the important commercial trades in agriculture. Growing of this cutflower has emerged as a high tech activity under controlled climatic conditions inside the green house. Under cultivation, the crop is affected by several biotic stresses among which the most significant damage is caused by the root knot nematode, *Meloidogyne* species. These nematodes are polyphagous in nature and can be controlled only by integrated management practices. Infection of this nematode results in heavy loss to the crops both in quality and quantity. Infection of this

nematode can be easily recognized by the formation of prominent root galls at the infection site. Though, yield loss due to this nematode is difficult to predict, approximate yield loss due to this nematode has been predicted by many authors in various crops. Nagesh and Reddy (2000) estimated the yield loss in gerbera due to *Meloidogyne incognita* infection as 31.1%. Another important biotic stress to which the crop exposed is the fungus, *Fusarium oxysporum* f. sp. *gerberae*. More than 70% of major crop diseases are caused by fungi and cause significant yield loss in most of the horticultural crops (Agrios, 2005). Controlled climatic condition in the green house favours the development of *F. oxysporum* in gerbera and cause severe economic loss to the farmers

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(Rajendran et al., 2014), which results in early death of the plant.

Presence of nematodes even breakdown the resistance of the plants to *Fusarium* infection (France and Abawi, 1994). Intensity of the damage caused by the nematode-fungus disease complex is more severe in plants than their separate infection (Jonathan and Gajendran, 1998; Jeffers and Roberts, 2003). Generally, root knot nematode-fungus interaction is considered to be one of the important factors responsible for the crop reduction and very little work has been reported on the nematode-fungus interaction in gerbera. This research was proposed to study the intensity of damage caused due to nematode-fungus disease complex in gerbera.

## MATERIALS AND METHODS

### Source and identification of root knot nematode associated with gerbera

Inoculum of root knot nematode was obtained from the nematode infected gerbera field at Coimbatore, Tamil Nadu, India. Root knot nematode egg masses were extracted from the roots of the gerbera. For the extraction, galled roots of the plants were collected and washed in water to remove the adhering soil particles and the protruding egg mass in the galls were collected under microscope with the needle. The collected egg masses were placed in distilled water for hatching. After 24 - 48 h, the entire eggs were hatched into juveniles and the freshly hatched juveniles were inoculated into one month old tomato plants cv. Co 3, planted in 5 kg mud pots filled with sterilized pot mixture at one juvenile per g soil (5000 juveniles / pot). After 30 days of inoculation of nematodes into tomato plants, eggs were extracted from tomato roots with the above mentioned procedure and used for the interaction studies.

Meanwhile, root knot nematode females collected from the gerbera roots were processed for perineal pattern to confirm the species of root knot nematode associated with the plant.

### Preparation of perineal pattern of root knot nematode

Matured *Meloidogyne* females were teased from the root galls and placed on a glass slide. They were cut at the neck region and body tissues were gently pushed out. The cuticle was placed in 45 per cent lactic acid to facilitate further cleaning. The cuticle was carefully trimmed so that 5-10 times the perineal area (area near to vulva) was retained. This was transferred to a fresh drop of glycerol and examined under microscope.

### Source and identification of *Fusarium* species associated with gerbera

Inoculum of *Fusarium* was obtained from the wilt infected gerbera plants collected at Coimbatore, Tamil Nadu, India. For the isolation of *Fusarium* species, a small section of the infected root (5-6 mm) tissues were cut and placed on Potato Dextrose Agar (PDA) medium with an antibacterial agent (streptomycin sulfate). The plate was incubated for 2-4 days. Conidial culture was prepared from the specimen to confirm the species.

### Preparation of conidial culture

The sporulated hyphae was scrapped from the PDA medium and

placed on a cavity slide. Few drops of water were added to the slide and mixed well with the hyphae. The slide was checked under microscope to observe the conidial characters.

### Nematode-fungus interaction in gerbera

Uniform sized, healthy tissue culture plants of gerbera var. Palmbeach obtained from Spic Biotech, Coimbatore, India were planted in 5 kg pots filled with sterilized pot mixture (red soil: sand: Farm Yard Manure (FYM): 2:1:1). One month after planting, the plants were inoculated with the fungus, *F. oxysporum* and the nematode *M. incognita* as mentioned in Table 1 at the rate of 50 ml conidial suspension (Ramamoorthy et al., 2002; Ramyabharathi and Raguchander, 2014) per pot and 5000 second stage juveniles of nematode per pot. Microconidial suspension of the fungi was prepared by pouring 20 ml of sterile distilled water in each Petri plate. Concentration of microconidia was adjusted to 1000 conidia/ml. Nematode and fungus inoculation was made by carefully adding the homogenous suspension of the two pathogens at the root zone of the plants.

Experiments were laid out in completely randomized design with four replications during the months of April to May, 2013 and repeated during the months of July to August, 2013; October to November, 2013 and December 2013 to January 2014 in the greenhouse at Tamil Nadu Agricultural University, Coimbatore, India maintaining the temperature range of 24 - 32°C.

Evaluations were performed 45 days after inoculation. Measurements were made on the plant growth parameters (shoot length and weight; root length and weight) and yield parameters: number of flowers per plant; flower diameter (cm) and stalk length (cm). Observations were made on the root population of nematode viz., number of females per g root, number of egg mass per g root and gall index of 1-5 scale (Gall index: 1=no galls; 2=1-25% galls; 3=26-50% galls; 4=51-75% galls; 5=76 -100% galls per root system) (Taylor and Sasser, 1978). Nematode population in soil was processed as per the sieving method of Cobb and Modified Baermann funnel technique. Per cent wilt incidence due to fungus was assessed using number of wilt infected plants /total number of plants taken for observation.

Data were analysed using analysis of variance (ANOVA). In the experiments that were repeated, since the error variances were similar, the analysis was performed on pooled data. Treatment means were compared and critical differences (CD) was calculated at P=0.05 to test for significant differences between treatments (T) (Panse and Sukhatme, 1978).

## RESULTS

### Identification of *Meloidogyne* species associated with gerbera

*Meloidogyne* species collected from gerbera were identified by the cuticular markings in the perineal area of the matured female. All the root knot nematode species observed were with high dorsal arch which were flattened at the top (Plate 1). This confirmed the species as *M. incognita*.

### Identification of *Fusarium* species associated with gerbera

The species was identified based on the morphological characters. Observation under microscope revealed small, oval shaped, single or bicelled microconidia and



**Table 1.** Interactive effect of *Meloidogyne incognita* and *Fusarium oxysporum* in Gerbera.

Treatments	Plant growth parameters							Nematode population				Per cent wilt incidence
	Shoot		Root		Stalk length (cm)	Flower diameter (cm)	No. of flowers/plant	Root population			Soil population	
	length (cm)	weight (g)	length (cm)	weight (g)				No. of females/g root	No. of egg mass/g root	Gall index (0-5 scale)	No. of juveniles/250 cc soil	
Nematode alone	31.85	35.70	19.93	31.88	37.88	8.20	2.00	46.75 (43.17)	22.75 (28.42)	5 (12.92)	347.5 (18.95)	6.5(2.54)
Fungus alone	31.60	38.13	20.78	41.08	37.95	8.70	1.75	0 (4.05)	0 (4.05)	0 (4.05)	0(4.05)	74.5 (8.62)
Concomitant inoculation of nematode and fungus	24.75	17.78	14.18	11.88	29.10	7.38	1.50	34.5 (35.95)	20.75 (27.07)	3.5 (10.75)	283.75 (16.88)	83.25 (9.11)
Nematodes 15 days prior to fungus inoculation	22.23	9.08	12.35	8.35	27.38	6.45	1.25	42.25 (40.53)	24 (29.30)	4 (11.48)	320.5 (17.53)	95.5 (9.76)
Fungus 15 days prior to nematode inoculation	28.65	23.20	18.28	28.63	32.45	7.43	1.75	20.5 (26.87)	10.75 (19.05)	3 (9.97)	249.75 (16.28)	74.25 (8.61)
Uninoculated control	35.50	43.47	22.90	46.81	46.93	9.35	3.50	0 (4.05)	0 (4.05)	0 (4.05)	0 (4.05)	0 (0.70)
SEd	1.04	0.88	0.82	0.57	0.50	0.24	0.48	1.91	1.57	0.29	2.79	1.18
CD (0.05)	2.19	1.84	1.72	1.20	1.05	0.51	1.01	4.02	3.29	0.61	5.85	2.49

\*Pooled data of four pot culture experiments carried out in the green house. Figures in the parentheses in nematode population analysis are square root of  $X+0.5$  transformed values; figures in parentheses for wilt incidence are arcsine transformed values. Gall index: 1=no galls; 2=1-25 % galls; 3=26-50% galls; 4=51-75 % galls; 5=76 -100 % galls per root system. SEd: Standard error difference; CD: Critical Differences.

hyaline, multicelled macroconidia with 3 septation which were sickle shaped with knobbed base at one end. This confirmed the species as *Fusarium oxysporum* (Plate 2).

#### Effect of individual inoculation of *M. incognita* and *F. oxysporum* on galls and wilt incidence

Nematode and fungus, when inoculated individually caused significant reduction in the plant growth parameters of gerbera when compared to untreated control (Table 1). Highest gall index of 5.0 and wilt incidence of 6.5 per cent was observed with the inoculation of nematodes alone to the pots. Fungus inoculation into the plants showed no galls in their roots, with however, 74 per cent wilt prevalence.

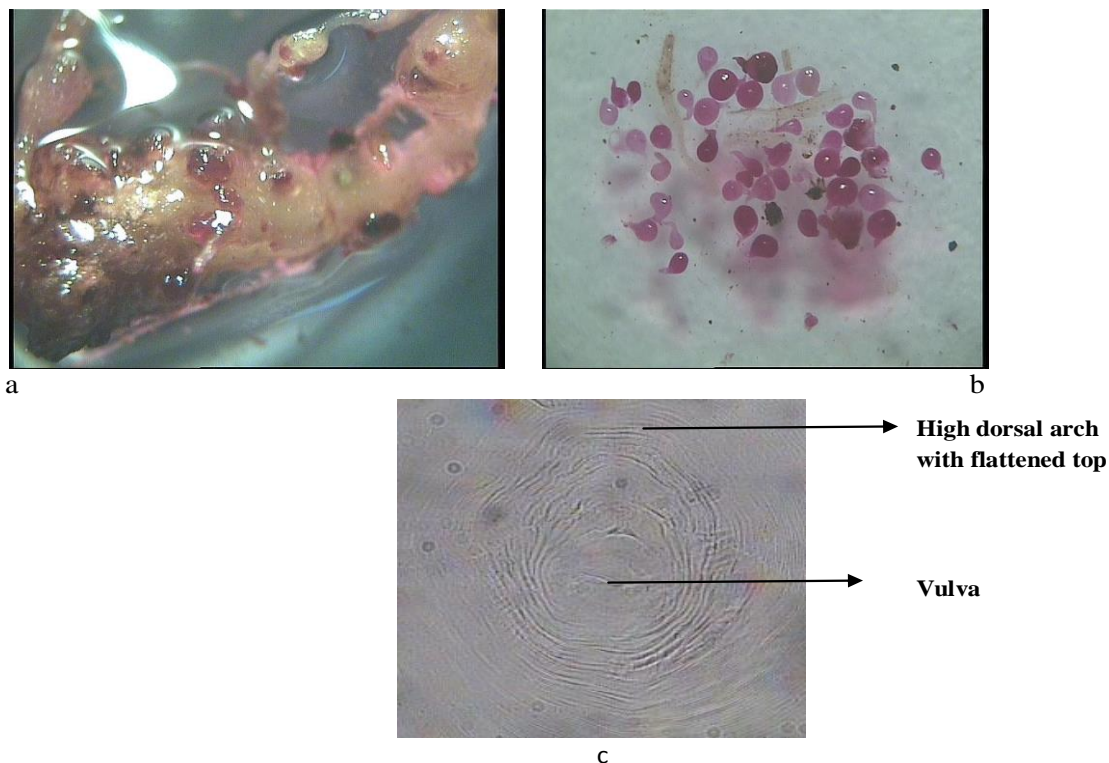
#### Effect of *F. oxysporum* and *M. incognita* on *Fusarium* wilt incidence

Initial symptoms of chlorosis and wilt incidence were observed as early as 7 days after inoculation of the fungus. In the sequential and concomitant inoculation of nematodes and fungus, wilt incidence was higher than with the fungus alone. Presence of nematode contributed to the early onset of wilt symptom which resulted in more stunting of plants. Sequential inoculation of nematodes 15 days prior to fungus significantly increased the severity of wilt incidence to 95.5 per cent followed by the concomitant inoculation of nematode and fungus which showed 83.25 per cent of wilt incidence indicating that nematodes predispose plants to infection by fungus and

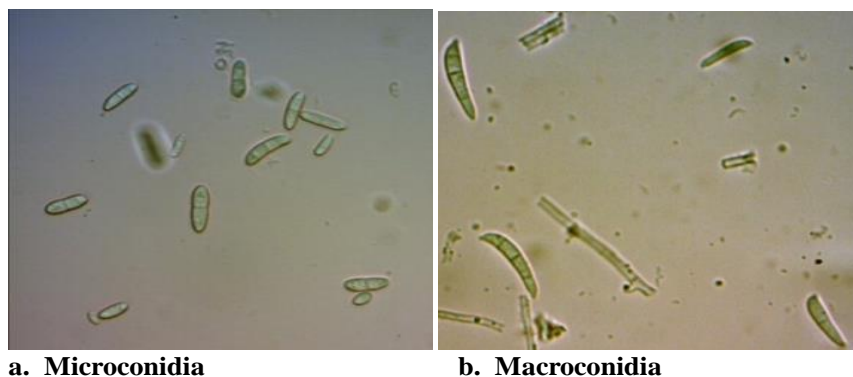
aggravate the disease incidence which ultimately leads to plants death.

#### Effect of *F. oxysporum* and *M. incognita* on the severity of root galls

Concomitant and sequential nematode and fungus inoculation resulted in a significant reduction of the gall index. Gall index of 4.0 was observed when the nematodes were inoculated 15 days prior to the fungus. The index was reduced with concomitant and sequential inoculation of fungus 15 days prior to nematodes. Severity of root galling diminished as the *Fusarium* density increased due to rotting of roots. This reduced the reproduction potential of the nematode. Similarly,



**Plate 1.** a. Root galls of gerbera; b. Root knot nematode females collected per g of gerbera root; c. Perennial pattern of root knot nematode, *M. incognita*.



**a. Microconidia**

**b. Macroconidia**

**Plate 2.** Conidial structures of *Fusarium oxysporum*.

lower nematode female density in roots and juvenile population in the soil were observed in the sequential inoculation of fungus 15 days prior to nematodes followed by the concomitant inoculation of nematodes and fungus.

## DISCUSSION

Interaction between nematodes and fungus have been reported previously in various crops viz., muskmelon (Bergeson, 1975), tomato (Sidhu and Webster, 1977),

*Vigna unguiculata* (Harris and Ferris, 1991), betelvine (Jonathan et al., 1996) and banana (Jonathan and Gajendran, 1998). Studies observed from the interaction in various crops revealed that nematodes predisposed the plants to the secondary infection by the fungal pathogens which were in agreement with the present study. Concomitant and sequential inoculation of nematodes and fungus resulted in the aggravation of wilt disease severity in gerbera compared with their individual inoculation. This was in accord with the study conducted by Jonathan and Gajendran (1998) in banana cv. Rasthali, where they observed that the incidence of

panama wilt disease was severe with the sequential inoculation of *M. incognita* followed by the fungus, *F. oxysporum* f. sp. *cubense* and concomitant inoculation of the two pathogens. Breakdown of resistance to *Fusarium* by prior infection with *Meloidogyne* has been reported in chickpea (Sharma et al., 1992), cotton, cucumber (Fritzsche et al., 1983), soybean, tomato (Bowman and Bloom, 1966; Sidhu and Webster, 1977). Severe wilt incidence due to fungus was observed in tobacco plants pre inoculated with nematodes (Porter and Powell, 1967). Nematode activities in roots modify roots physiology and morphology (Sankari Meena et al., 2011) which make the plant more vulnerable to the infection by secondary pathogens (Mayol and Bergeson, 1970). El-Shawadfy et al. (1988) suggested that presence of fungus did not altered the invasion of nematodes into the roots but affected the development of nematode females and severity of galls which might be due to the invasion of giant cells of nematodes by the *Fusarium*. This ultimately restricts the nematode reproductive potential in the plant. Moreover, toxic metabolites produced by the fungi may also reduce the egg hatching of nematodes and immobilize the second-stage juveniles of them (Fattah and Webster, 1989). These findings were confirmed by the present study where reduced nematode population and gall index with increased wilt incidence were observed with concomitant nematode and fungus inoculation followed by sequential pathogens inoculation. Thus it was proved from the present study that presence of nematode paves way for the early entry of the fungus into the plants which aggravate the wilt disease severity than the individual inoculation of nematodes and fungus.

### Conflict of interests

The author(s) did not declare any conflict of interest.

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