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Full Length Research Paper

Effect of neem (*Azadiracta indica*) aqueous extract on *Casuarina equisetifolia* seed germination, growth of *Frankia* and some rhizospheric microorganisms

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Aqueous neem (*Azadiracta indica*) leaf extract was tested for its effect on *Casuarina equisetifolia* seed germination capacity, growth of *Frankia* and on some dominant rhizosphere microorganisms under casuarinas grown in soil contaminated with sewage water. Analysis was carried out to determine the main micro- and macroelement content of different extract amounts. The addition of extract amount between 10- 20 µl per 10 ml medium (1 - 2 µl ml^{-1}) selectively stimulated *Frankia* growth and inhibited other non- beneficial bacteria and fungi in the rhizosphere such as *Bacillus subtilis*, *Drechslera* sp and *Curvularia lunata*. Slight increase was observed in *Casuarina* seed germination capacity with 1- 2 µ ml^{-1} of the extract, but with no significant differences, indicating at least no negative effect of the extract on seed germination. According to the results and as the roots and leaves of both *Casuarina* and neem release their active components in soil, and according to previous recommendations, we suggest a significant synergistic effect between *Casuarina*, rhizosphere microorganisms and neem trees for the benefits of *Casuarina* nodulation, growth performance and nitrogen fixation.

Key words: Neem, *Casuarina*, aqueous extract, *Frankia*, rhizospheric microorganisms.

INTRODUCTION

Neem (*A. indica*) plant extracts have been used for its antibacterial, antifungal and insecticidal activities in addition to improvement of plant seed quality and emer-gence of plant seedlings (Nwachukwu and Umechuruba, 2001; Gopal et al., 2007; Zhang et al., 2010). The crude neem extract exhibited antimicrobial activity against both Gram- positive and Gram- negative bacteria, and provided a physical barrier to conidial germination while its limonoids and other compounds provided fungicidal activity (Coventry and Allan, 2001). Neem oils contain different quantities of limonoids and the major neem compounds azadirachtin, nimbin and salannin (Coventry and Allan, 2001).

On the other hand, free radicals were considered a major cause for aging of *Casuarina* seeds that have the capacity for repairing and protection against its deleterious effects (Woodstock, 1967). Pre-coating of *Casuarina equisetifolia* seeds with neem leaf powder can sustain the activity of the enzymes amylase, catalase, peroxidase and superoxide dismutase, that maintain the seed germination potential (Umarani and Vanangamudi, 2005).

Actinorhizal plants are those nodulated by the symbiotic nitrogen- fixing actinomycete *Frankia* (Dawson, 2008). These plants are very important for increasing soil N content, land reclamation, agroforestry, as timber trees and many other uses (Schwencke and Caru, 2001; Dawson, 2008; Sayed, 2011). Growth of *Frankia* is slow and result in low biomass production and some growth-promoting factors were suggested to stimulate better growth (Quispel et al., 1983; Schwencke, 1991; Girgis and Schwencke, 1993).

The objectives of this study is to evaluate the impact of the aqueous extract of neem leaves *in vitro* on the rhizosphere of *C. equisetifolia*. This was carried out by measuring growth of some *Casuarina*- infective *Frankia* strains, and some dominant rhizospheric microorganisms

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Table 1. The tested *Frankia* strains.

Strain	Source	Reference
HFP020203 ($HFPCcI_3$)	*Casuarina equisetifolia*	Zhang et al., 1984
UGL020604	*Casuarina equisetifolia*	Sempavalan et al., 1996
ORS021001	*Casuarina junghuniana*	Diem et al., 1983

that were treated with neem extract. The effect of extract was tested also on *C. equisetifolia* seed germination capacity.

MATERIALS AND METHODS

Preparation and analysis of *A. indica* extract

Green leaves of *A. indica* were collected from ten- year- old trees inside the South Valley University campus at Qena (Egypt). Samples were collected in polyethylene bags and transferred immediately to the laboratory. Leaves were washed thoroughly with tap water and dried at 80°C for 48 h. Dried samples were then ground in a tissue grinder (IKA A 10, Germany) to fine powder.

Extracts were prepared by homogenizing 50 g of dry leaf powder in distilled water and completing to 1l with water. The homogenate was filtered through Whatman# 1 filter paper and filter- sterilized by passing through 0.2 μm filter. The following criteria were determined in the filtrate:

1. pH by using AS- 501 pH Analyzer (STEM corporation, England).
2. Conductivity and total dissolved salts (TDS) using a 'Checkmate 90' meter equipped with conductivity/ TDS sensor (Corning®, USA).
3. Sodium and potassium using a Genway® flame photometer, UK (Williams and Twine, 1960).
3. Iron, manganese, zinc, magnesium, copper, calcium, and phosphorus using Buck Scientific INC 210– Atomic Absorption Spectrophotometer (AAS) after the extraction of the filtrate with 0.005 M diethelyne- triamine- pentaaceticacid (DTPA) buffered at pH 7.3 (Lindsay and Norvell, 1978).

Frankia strains and cultural conditions

Three *Frankia* strains (Table 1) were grown in 20 ml of propionate-BAP medium (Murry et al., 1984) in sterile 50 ml bottles. After four weeks of incubation at 28°C, hyphae were washed by filtration through 0.2 µm membrane filter and re- suspended in sterile distilled water. Cultures were homogenized in a glass tissue homogenizer and parts of suspensions were sonicated for one minute using an ultrasonicator (Dr- Hilscher, Germany) as described by Sayed and Wheeler (1999). Growth was measured as protein concentration by using Coomassie® blue assay (Bradford, 1976). Optical density of the growing cultures was measured at 595 nm on a "SPECTRONIC® GENESYS™ 2PC" spectrophotometer, Spectronic Instruments, USA. Cultures were then diluted with sterile distilled water to give a final protein concentration of 5.5 µg ml^{-1} in the medium (Hooker, 1987).

Effect of neem extract on *Frankia* growth

Two sets of three tubes each containing 10 ml BAP medium (Murry et al., 1984) were inoculated with the tested *Frankia* strains (Table 1). Each tube, was inoculated with 1 ml of sterile distilled water containing 5.5 µg of the tested *Frankia* protein. Tubes were amended with the following amounts of sterilized neem aqueous extract: 0.0 (control), 10, 15, 20, 30, 50, 70, 100, 300, 500 and 1000 µl. Cultures were incubated at 28°C for 4 weeks and tubes were shaken vigorously once per day for homogeneity. By the end of the incubation period, the contents of each tube were washed twice by centrifugation at 4000 rpm in a "Biofuge, Primo R, Germany" centrifuge for 15 min with sterile distilled water. *Frankia* protein content was determined as aforementioned (Bradford, 1976).

Rhizosphere microorganisms from *Casuarina* growing in sewage- polluted soil

Three soil samples were collected (each is a composite of several cores, 0- 10 cm depth) from under *Casuarina equisetifolia* trees growing in EL- Salahia, Qena, Egypt. Soil of this area is regularly irrigated with partially- treated sewage water. Samples were mixed together and their solutions were prepared by suspending a known weight of soil in a known volume of water and stirring continuously for 30 min (Case and Johnson, 1984). The resulting suspension was used for the isolation of different soil microorganisms after preparing serial dilutions from the original solution. Plate count method was used, for the isolation of fungi, by inoculating 1 ml of each dilution into sterile petri plate containing Czapek's agar medium (Smith and Dawson, 1944) with 1/ 3 x 10^4 rose bengal and 0.05 mg ml^{-1} chloramphenicol as antibacterial agents. The plates were incubated at 28°C for 7- 10 days and the developed fungal colonies were counted (Domsch et al*.,* 1980).

Isolation of bacteria was carried out using spread plate method on two different media; nutrient agar and nitrogen- free agar medium supplemented with sucrose or mannitol (Seeley and Van Demark, 1981). The nutrient agar medium was employed as a general purpose medium for isolating most of soil bacteria while the nitrogen- free medium was for isolating the free nitrogen- fixing bacteria in the affected soil. Plates of these solid media were inoculated with 0.1 ml of different soil dilutions. The isolated bacteria with the highest colony counts were identified (Bergey's Manual® of Systematic Bacteriology, 1986).

Effect of the extract on some dominant rhizospheric bacteria and fungi

Dominant fungi and bacteria (i. e. of the highest colony counts in the isolation plates) were grown as pure cultures on the appropriate medium. The used media were nutrient broth for *Bacillus* and *Azotobacter* and Czapeck's agar for the isolated fungi. The growth media of these dominant bacteria and fungi were supplemented with the effective concentrations of aqueous *A. indica* extract (i.e. 10, 15 and 20 µl) in addition to 0.0 (control) treatment. Growth of these dominant bacteria and fungi was measured as protein content and dry weight, respectively.

Effect of the extract on *Casuarina* seed germination

Seeds were collected from the same *Casuarina* plants inside South

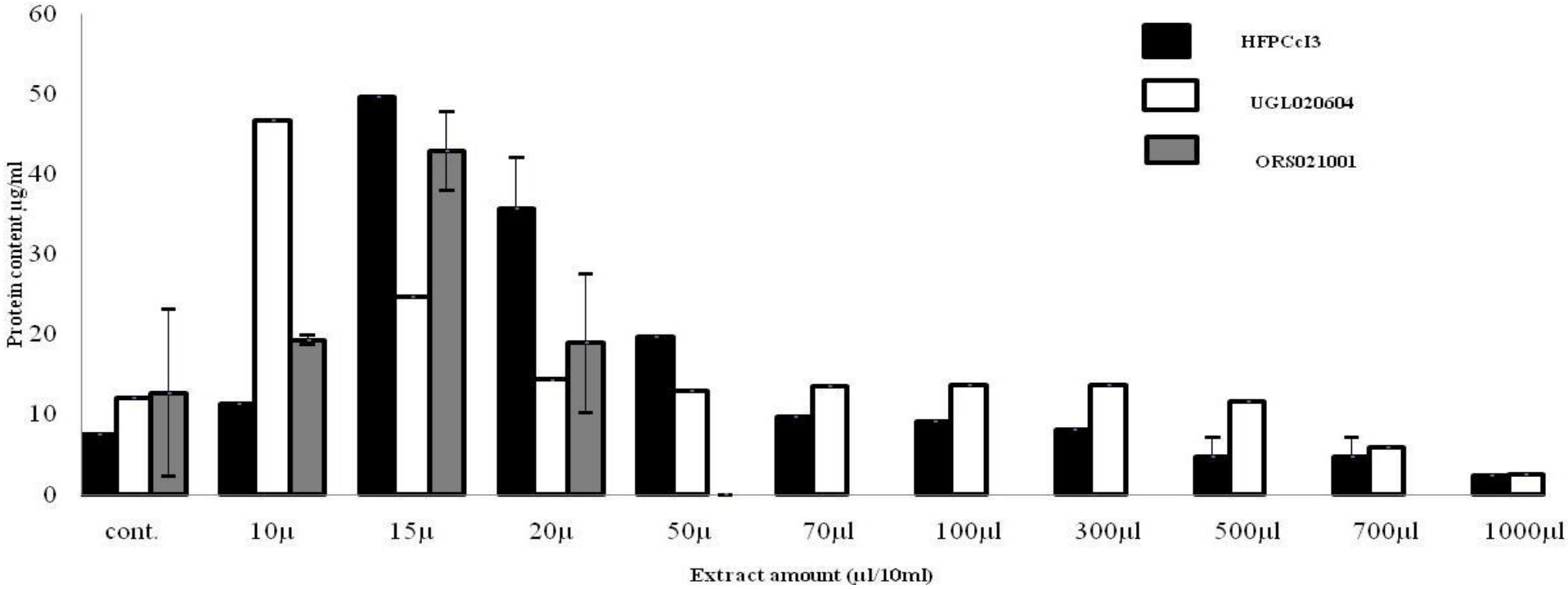

Figure 1. Effect of different amounts of *A. indica* aqueous extract on growth of some *Frankia* strains.

Valley University as above. The collected seeds were surface sterilized with H_2O_2 (30%) for 3- 5 min and rinsed several times with sterile distilled water (Sayed, 1995). Replicates of fifty seeds each were germinated in sterile petri plate containing water agar medium supplemented with the tested quantities of neem extract as indicated earlier. Plates were left to germinate in the dark for 15 days at 28°C. The percentage of seed germination was then calculated and compared to control.

Statistical analysis

The results were statistically analyzed using the least significant differences test (L.S.D) on PC- STAT program (version 1A) coded by Rao, M.; Blane, K. and Zannenberg, M, University of Georgia.

RESULTS

Analysis of the extract and its effect on *Frankia* growth

There were no significant differences in pH, conductivity and TDS values between all treatments and control (data not shown). The values of some elements, added to *Frankia* medium by the extract, such as Zn, Fe and Mn were low compared to elements such as Ca and Mg when 10, 15 and 20 µl of extract were added to the medium. For extract amounts between 10 and 20 µl, the following percentages of elements were added to the medium: calcium (15- 31), sodium (0.003- 0.006), magnesium (4- 8), potassium (0.0007- 0.0014), iron (0.004- 0.007), manganese (0.01- 0.02), copper (0.34- 0.68) and zinc (0.012- 0.025). Percentages were calculated considering that element concentration in BAP medium= 100%. Higher extract amounts (i.e. 50 to 1000 µl) amended the BAP medium with very high percentages (data not shown) with increased toxicity that is reflected by a highly reduced *Frankia* growth (Figure 1).

Growth of all the tested *Frankia* strains increased significantly with 10, 15 and 20 µl of extract as shown in Figure 1. Growth of *Frankia* strain HFP020203 increased significantly with 10, 15 and 20 µl of extract to 1.5, 6.6 and 4.7 fold

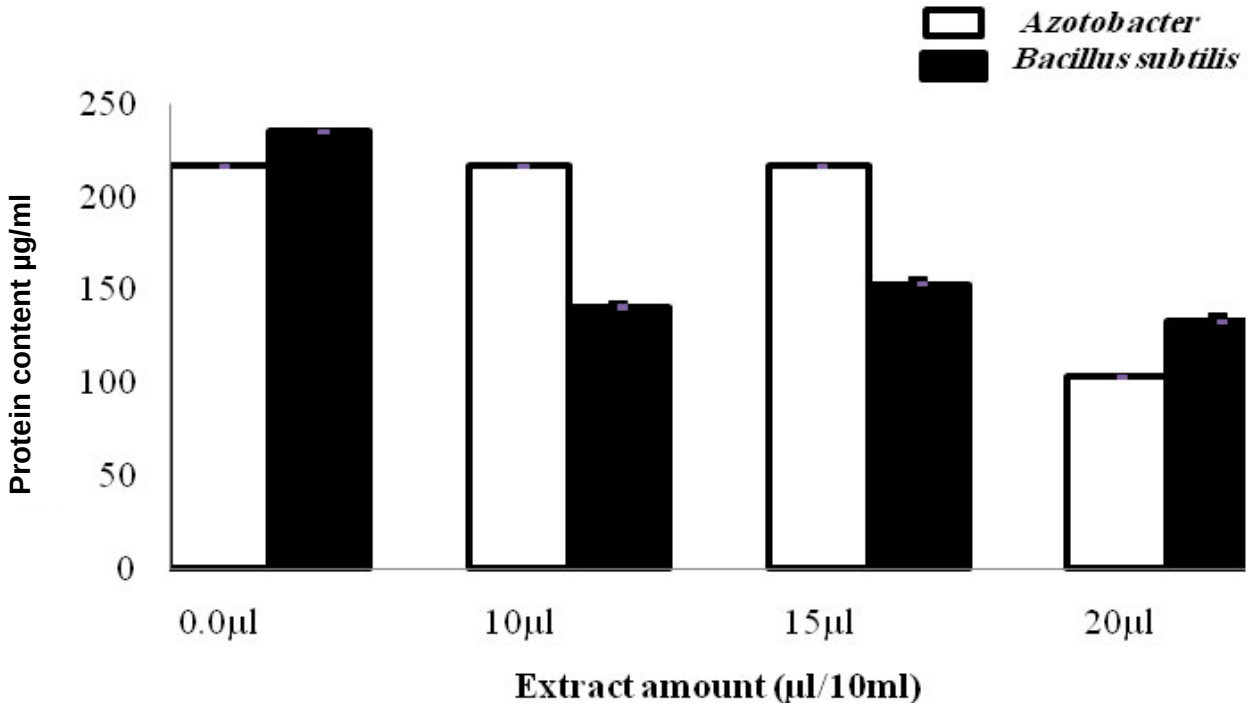

Figure 2. Effect of aqueous neem extracts on *Azotobacter sp*. and *Bacillus subtilis*.

compared to control (P= 0.01). For strain UGL020604, the highest growth values (i.e. 3.8 and 2.1 fold, compared to control) were recorded with 10 and 15 µl of extract. Inhibition of *Frankia* occurred when grown with higher extract amounts (700 to 1000 µl) as shown in Figure 1.

Effect of the extract on growth of dominant rhizospheric microorganisms

Two bacterial strains (*Bacillus subtilis* and *Azotobacter* sp.) and two fugal strains (*Drechslera* sp and *Curvularia lunata*) were the most dominant in *C. equisetifolia* rhizosphere in the polluted soil. Growth of *B. subtilis* was reduced significantly by 40% with the addition of 15 µl of *A. indica* extract compared to control (Figure 2). Growth of *Azotobacter* sp was not affected greatly with 10 and 15 µl while with 20 µl there was a significant decrease in growth (53%) compared to control.

On the other hand, growth rate of both *Drechslera* sp and *Curvularia lunata* decreased gradually by increasing *A. indica* extract amount to 20 µl with higher inhibition for the former organism (Figure 3).

Effect of the extract on *C. equisetifolia* seed germination capacity

There were no significant differences between treatments (P= 0.05) indicating no negative effects for the extract on *Casuarina* seed germination.

DISCUSSION

Frankia form clusters of vesicles inside the plant root nodule cells or in culture under nitrogen- limiting conditions that represent the site of nitrogen fixation (Tjepkema et al., 1986; Benson and Silvester, 1993). In some *Frankia* strains, calcium is required for both vesicle development and subsequent nitrogenase activity (Tisa and Ensign, 1987).

In the current study, extract of *A. indica* leaves significantly increased growth of the three tested *Frankia* strains with the addition of 10, 15 and 20 µl of the extract (Figure 1). These amounts of the extract amended the medium with 15, 23 and 31% Ca and 4, 6 and 8% Mg more than the original concentrations in the medium. *Frankia* growth increased with elevated calcium concentrations up to 0.25 g l^{-1} in culture medium in a previous study (Abd El- Karim, 2004). The same effect of *Frankia* stimulation was obtained when cultures amended with *Casuarina* leaf litter extract that contained variable concentrations of different elements including calcium (Sayed et al., 2002). The host plant also release compounds that increase *Frankia* infective units and nodulation capacity including phenolics and fatty acids (Krumholz et al., 2003; Selim and Schwencke, 1995; Zimpfer et al., 2002).

Metal ions have been released into the environment over long period of time throughout many human activities (El- Enany and Issa, 2000). Several laboratories reported the toxicity of higher concentrations of different metal ion that affect *Frankia* and plant growth (Sayed et

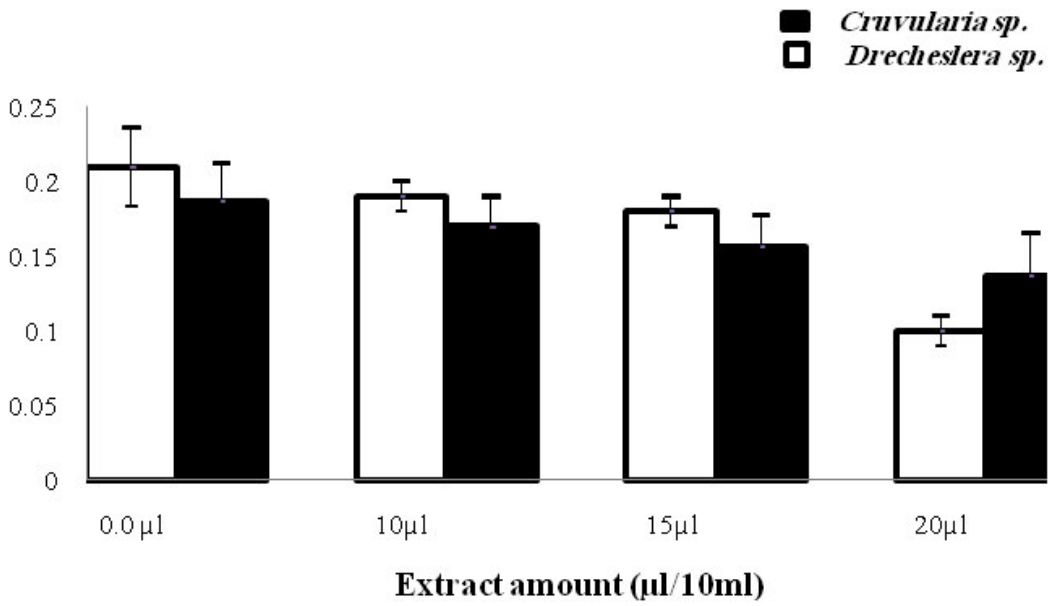

Figure 3. Effect of neem aqueous extract on *Drechslera* sp and *Curvularia lunata.*

al., 2000; Wheeler et al., 2001; Richards et al., 2002; Sayed, 2003; Markham, 2005; Cusato et al., 2007).

In the current study, growth of *Frankia* strains HFP020203 and UGL020604, was reduced by 68 to 79%, respectively, with the amounts of extract between 700 to 1000 µl (Figure 1).

This can be explained as a direct response to medium amendment with the toxic concentration of some elements such as Zn, Cu and Mn by the increased amounts of neem extract (Figure 1). Therefore, the pronounced growth stimulation, of the lower extract amounts, may be principally due to the addition of increased amounts of Ca, Mg and other macronutrients while adding non- toxic concentrations of micronutrients.

Several "active components" from *A. indica* have demonstrated high efficiency against most pathogens (Khan and Wassilew, 1987). As fungicides, over 14 common fungal species are sensitive to *A. indica* preparations (Khan and Wassilew, 1987; Sai Ram et al., 2000). Pathogenic bacteria like *Staphylococcus aureus*, *Salmonella typhi* are also significantly suppressed by neem extract (Sai Ram et al., 2000) . Furthermore, the addition of 10% azadirachtin granules to soil significantly suppressed bacteria, fungi, actinomycetes and other microorganisms except *Azotobacter* (Gopal et al., 2007; Zhang et al., 2010). Our results showed that growth of *Bacillus subtilis* decreased significantly by increasing the added extract amount from 10 to 20 µl while *Azotobacter* growth was not affected until treated with 20 µl (Figure 2). The dry weight of *Drechslera* sp and *Curvularia lunata* was also reduced with the elevated extract amounts up to 20 µl (Figure 3). This indicates a selective effect of these extract amounts for stimulating beneficial microorganisms while inhibiting other harmful or pathogenic ones in the rhizosphere of *C. equisetifolia*. One of the possible reasons for increased nodulation, by releasing compounds from actinorhizal plants, are growth stimulation of *Frankia* and helper organisms while inhibiting *Frankia* competitors in soil (Gauthier et al., 2000; Dawson, 2008). Therefore, neem extract may participate at least by keeping other microorganisms away from the competition with *Frankia*. Other roles such as supplying nutrients that enhance *Frankia* growth and nodulation capacity require more investigation.

On the other hand, seed propagation of *Casuarina,* as with many other plants, requires rapid and uniform germination to achieve efficient nursery production (El- Lakany and Shepherd, 1983). Seeds of *Casuarina* normally have germination percentages between 10 to 50% due to the presence of damaged or empty seeds in the seed lots and some other factors (Boland et al*.,* 1996; Sivakumar et al*.,* 2007). Leaves of *A. indica*, were reported among other plants, to have insecticidal pro-perties (Gopal et. al., 2007). Treatment of *C. equisetifolia* seeds with neem leaf powder was also capable of maintaining the seed germination potential and seedling viability (Umarani and Vanangamudi, 2005).

Although *C. equisetifolia* seed germination slightly increased with increasing the amount of *A. indica* extract from 10 to 20 µl (data not shown), but there were no significant differences with the control treatment. This indicates no beneficial or harmful effect on seeds that is supported by the clearly documented effect on long storage of seeds (Umarani and Vanangamudi, 2005; Gopal et al., 2007).

In summary, growth of *Frankia* strains is enhanced by

the use of 1- 2 µl ml^{-1} of *A. indica* aqueous leaf extract. The same stimulatory amounts of leaf extract inhibited some non- beneficial and pathogenic bacteria and fungi that are dominant in the rhizosphere of *C. equisetifolia* such as *B. subtilis* , *Drechslera* sp. and *Curvularia lunata*. These organisms were isolated from under casuarinas grown in sewage- contaminated soil. Therefore, the addition of neem extract in the rhizosphere may be a method for biologically controlling both pollution and disease- causing microorganisms, and stimulation of the beneficial organisms in such environments. Neem extracts and cakes were previously nominated for improving soil- N content and reducing the emission of green house gases from soil (Mendez-Bautista et al., 2010).

In conclusion, as leaf litter and root exudates of both neem and *Casuarina* release the required active components in soil, we suggest a synergistic interaction between *Casuarina*, its rhizosphere microorganisms and neem for better plant performance.

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