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EFFECT OF PLANT GROWTH PROMOTING MICROORGANISMS FROM RHIZOSPHERE OF *PIPER NIGRUM* L.

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ABSTRACT

Six fungal isolates were selected from the rhizosphere of black pepper (*Piper nigrum* L.) plants and were tested in vitro for their plant growth promoting (PGP) abilities in the soil. The species of *Aspergillus* selected were tested for the siderophore production, indole acetic acid (IAA) production, catalase enzyme activity and biological control of pathogens through antagonism. The results indicated high degree of PGP ability in case of *Aspergillus niger* followed by *A. flavus* str1 with respect to all parameters. *A.niger* (12 U mL^{-1}) and *A. flavus* str1 (10 U mL^{-1}) showed higher siderophore production capabilities were able to produce indole acetic acid (IAA) in the range of 710µg mL^{-1} *A. niger* with a high catalase activity of 55.69 units mL^{-1} exhibited good biocontrol abilities against both *Fusarium equiseticus* (15.6 mm) and *Mucor* spp. (11.6 mm) suggesting that *A. niger* could be considered as a potential plant growth promoting fungi (PGPF).

KEY WORDS: Plant growth promoting fungi (PGPF), siderophore, indole acetic acid (IAA), catalase activity, biocontrol, *A. niger*.

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INTRODUCTION

Black pepper is one of the most widely used spices in the world and India is one of the largest exporters of pepper. The pepper plant, (*Piper nigrum* L.) has been an important crop plant cultivated for millennia in the Malabar regions of Karnataka, Kerala and some regions in Tamil Nadu, India. The complexity of the soil system is determined by the numerous and diverse interactions among its physical, chemical, and biological components, as modulated by the prevalent environmental conditions[1]. In particular, the varied genetic and functional activities of the extensive microbial populations have a critical impact on soil functions, based on the fact that microorganisms are driving forces for fundamental metabolic processes involving specific enzyme activities[2]. Many studies have demonstrated that soil-borne microbes interact with plant roots and soil constituents at the root–soil interface[3]. The great array of root–microbe interactions results in the development of a dynamic environment known as the rhizosphere where microbial communities also interact. Rhizobacteria inhabit plant roots and exert a positive effect ranging from direct influence mechanisms to an indirect effect and are termed PGPR. In the last few years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere[4]. The production of phytohormones, which are signal molecules acting as chemical messengers, by PGPR is now considered to be one of the most important mechanisms by which many rhizobacteria promote plant growth[5]. Similarly numerous fungal species can produce phytohormones[6]. The introduction of biotechnology products into agriculture has improved in order to increase yields and crop quality, and siderophores produced by several of the bacteria and fungi are being used to reduce the rhizospheric population of phytopathogenic fungi and bacteria. Siderophores bring about the inhibition of pathogens in the soil by sequestering iron from the pathogens, thus limiting their growth[7]. Siderophores are ferric-specific microbial iron-chelator compounds whose biosynthesis is regulated by the availability of iron in the surrounding medium and under conditions of high iron concentrations; the production of these compounds is repressed. Many microorganisms utilize an efficient system of utilizing iron present in form of ferric salts in the environment, [8,9,10]. Studies of microorganism siderophore producers have received much attention because of the clinical applications and potential utilization of these chelators in agriculture[11, 12].

Conventional strategies of disease control were replaced with the use of chemical fungicide. However, these fungicides affected soil fertility and the ecosystem. With root rot and spot, the efficacy of fungicides and plant genetic resistance is determined by the interaction of environmental and cultural conditions[13]. To overcome this problem, biocontrol agents have been and are being, investigated. The mechanism of disease reduction may involve antagonist activity, i.e., by siderophore- mediated competition that reduced in the availability of iron for the survival of pathogens which results in the exclusion of fungal pathogens in the rhizosphere. These beneficial siderophore-producing rhizobacteria suppress some soil-borne fungal pathogens[14], and there is convincing evidence to support a direct role of siderophore-mediated iron competition in the biocontrol ability[15]. Studies on biological nitrogen fixation have revealed the role of indole acetic acid (IAA) produced by rhizobacteria in increasing the absorption of nutrients by increasing the production of root hairs by the plant body[16]. It has been observed by scientists that the IAA production by soil fungi is enhanced by the release of tryptophan by the root exudates[17, 18, 19]. The ability to

synthesize IAA has been detected in many rhizobacteria as well as in pathogenic, symbiotic and free living bacterial species[20, 6, 22]. Over the past one hundred years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens[23]. The interactions between microorganisms and plant hosts can be complex. Interactions that lead to biocontrol can include antibiosis, competition, induction of host resistance, and predation[24]. Similar to plant growth promoting rhizobacteria, some rhizosphere fungi able to promote plant growth upon root colonization are functionally designated as 'plant-growth-promoting-fungi' (PGPF**),** which have been shown to trigger systemic resistance against various pathogens in cucumber plants, are non-pathogenic soil inhabiting saprophytes, have been reported to be beneficial to several crop plants not only by promoting their growth but also by protecting them from diseases, [24, 25]. In the present investigation efforts have been done to study the plant growth promoting rhizofungi from the rhizospheric soils of black pepper (*P. nigrum* L.) in the pepper cultivating regions of Karnataka, India. According to the available literature, fungal isolates have been tested for their capacity to produce siderophores, IAA, catalase enzyme activity and their ability as biocontrol agents.

MATERIALS AND METHODS

Isolation of rhizospheric microbes

The rhizospheric soils were collected from the different pepper growing regions of Karnataka, India, from four different regions of different pepper plants from plantations in Murunadu (12°18'E, 75°45'E), and were mixed together. Triplicates were taken for further analysis. Similar procedure was followed from the other two different pepper plantations from Birur (13°53'N, 75°58'E), and one home stead farm from Bangalore (12°58'N, 77°48'E), co-cultivated with coconut (*Cocos nucifera* L.) trees. The rhizospheric soil around the uprooted plants of black pepper which were mature, three years of age and in fruiting stage was collected for soil sampling to obtain variability in the microorganisms. Six different isolates of *Aspergillus* species isolated on Martin's raised Bengal agar media were selected for studying the production of siderophores, indole acetic acid and catalase enzyme. The cultures were maintained in their pure state on Sabaroud's dextrose agar (SDA) and were identified up to the species level, [26].

Siderophore assay

Chrome azurol S (CAS) assay media was used to detect siderophores produced by rhizofungi according to Schwyn and Neilands [27]. The plates point inoculated and colonies with orange or pink zones were considered as siderophore–producing strains. Assay in solid media was carried out in triplicate. The control plates of CAS-agar (uninoculated) were incubated under the same conditions as described above and no color change in the CAS-blue agar was observed after incubation periods of 1–14 days, [19, 15, 28]. The production of siderophores in solid CAS media was expressed in mm of zone produced after the period of incubation.

Cultures in CAS broth were incubated at 30°C with shaking (120 rpm) for 10 days until the culture had produced enough surface mat after 10 days, it was filtered using Whatman no.1 filter paper and the broth was assayed spectrophotometrically for the presence of catechol and hydraxamate type of siderophores by taking OD at 450 nm and at 500 nm respectively using ferric per chloride assay method, [12].

Production of IAA

Czapek dox broth containing 0.5% tryptophan was prepared, sterilized, cooled and inoculated with the given fungal cultures which were incubated for a period of 15 days at 28 ºC. Fully grown cultures were centrifuged at 3000 rpm for 30 min. the supernatant (5mL) was thereafter tested with Kovac's reagent to test

the presence of IAA in the broth cultures, [29, 30]. Further the cultures were tested with Salkowski's reagent; development of pink color indicated the production of IAA, [31].

Production and detection of catalase

The selected fungal cultures grown on czapek dox broth for a period of 7 days were tested for the activity of the catalase enzyme spectrophotometrically as described by Beers and Sizer[32] in which the disappearance of peroxide in the solution is followed at 240 nm. One unit decomposes one micromole of H_2O_2 per minute at 25°C and pH 7.0 under the specified conditions. 0.05 M Potassium phosphates, pH 7.0 0.059 M Hydrogen peroxide (30%) in 0.05 M potassium phosphate buffer at pH 7.0 was used as the reagent.

Mg enzyme mL^{-1} = A 240 × 0.667

The OD values were recorded and the time taken for the OD to reduce by 0.5 noted down. The activity of catalase calculated for each of the cultures[33] using the formula,

Units/mg (enzyme activity)

$$\text{Units/mg} = \frac{\Delta A_{240}/\text{min} \times 1000}{43.6 \times \text{mg enzyme/ml reaction mixture}}$$

Biocontrol of pathogens by Aspergillus species

Six species of *Aspergillus* and one species of *Fusarium, F. equiseticus* and *Mucor* were selected for studying the antagonistic effect of species of *Aspergillus* against the pathogens**.** All the cultures were maintained on Sabaroud's dextrose agar slants at 4 °C. Antagonistic properties of siderophore producing fungi, *Aspergillus* species were tested against these pathogenic fungi on SDA plates using a dual culture technique [34]. Five day old cultures of exponentially grown cultures of Aspergillus species were streaked 5 cm juxtaposed from the streak of the pathogen. The plates were incubated for 5 days at 28 C. The zone of inhibition of growth was calculated as the distance between the edges of the colonies of the two organisms. The zone of inhibition was recorded[15].using the formula: Inhibition (%) = (C–T)/(C) × 100, where ''C" is the maximum growth of the fungal mycelia under control conditions and T is fungal mycelia growth in dual culture. The organisms *F. equiseticus* and *A.niger* were identified up to molecular level with the help of genomic DNA sequencing.

RESULTS

Rhizospheric isolation

The isolation of fungi from the rhizospheric soil of Pepper plant (*Piper nigrum* L.) yielded several groups of fungal organisms of which six species of *Aspergillus* were selected and further studied for their role in the soil with respect to their plant growth promoting abilities. The species of *Aspergillus* selected were *Aspergillus flavus* str1*, A. flavus* str2*, A. clavatus, A. terreus, A. fumigatus*, and *A.niger* and were tested for their role as PGP fungi. The biocontrol capacity of *Aspergillus* species selected was tested for their antagonistic effect against two isolates *Fusarium* spp. and *Mucor* spp. (Table 1).

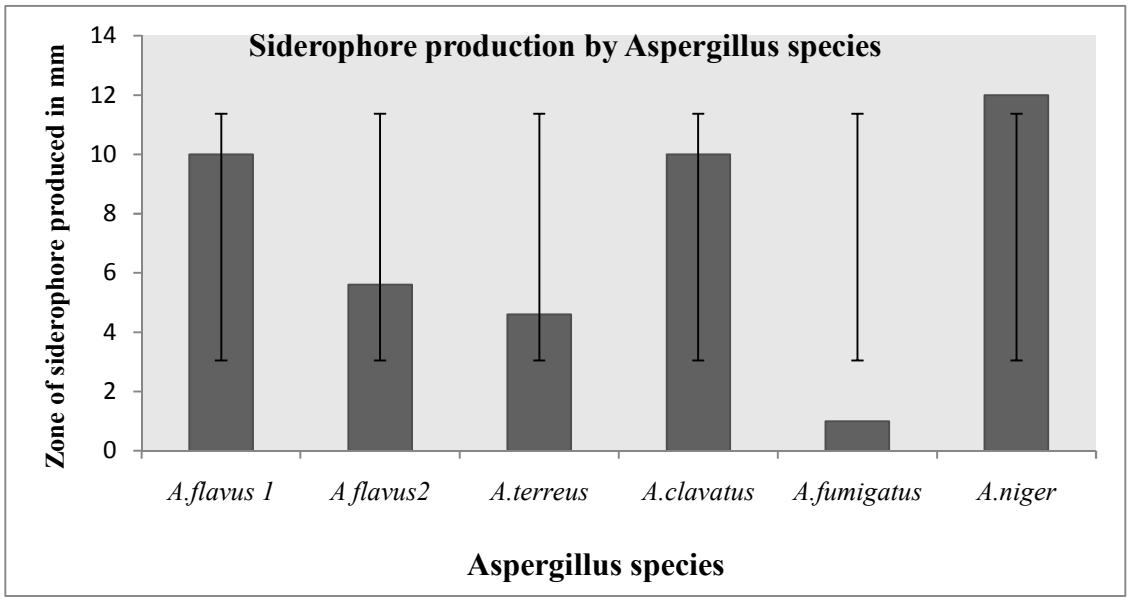
Table1
Fungi isolated from the rhizospheric soils of different samples of Black pepper (P.nigrum L.) plants

Sl. No.	Soil Sample	Geographical location	Soil type	Organisms isolated	Colony count (colony forming units in cfu g^{-1})
1	Murunadu Sample	Murunadu, Karnataka, India 12°18'N, 75°45'E	Black loamy clay	*A. ochraceous*	2×10^4
				A. flavus,	1×10^3
				Fusarium spp.	1×10^3
				Penicillium spp.	2×10^5
2	Beerur sample No.1	Beerur, Karnataka, India 13°53'N, 75°58'E	Red loamy	*Fusarium spp.*	1×10^5
				Cladosporium	1×10^5
3	Beerur sample No.2	Beerur, Karnataka, India 13°53'N, 75°58'E	Red loamy	*A. fumigatus*	1×10^4
				A.terreus	1×10^4
				Fusarium spp.	1×10^3
				A. flavus str 2	1×10^4
				Penicillium spp.	1×10^5
4	Bangalore Sample	Bangalore, Karnataka, India 12°58'N, 77°48'E	Red loamy	*A. niger*	7×10^4
				A. flavus	2×10^4
				A. clavatus	1×10^3
				Penicillium spp.	3×10^4

Siderophore production

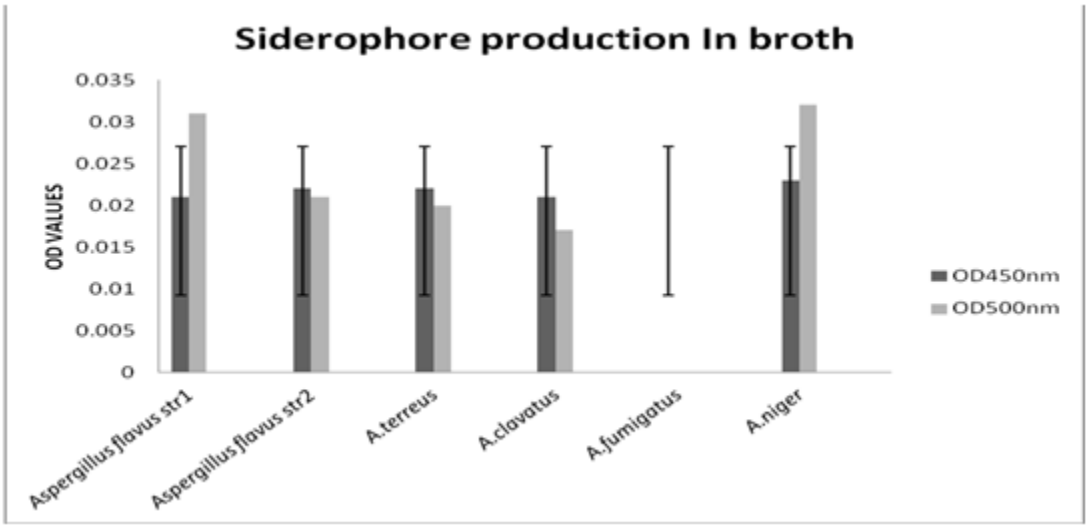
All the isolates of *Aspergillus* species chosen for the siderophore evaluation showed good production both on solid CAS agar media and in CAS broth media. It was seen that the maximum siderophore production was shown by *Aspergillus niger* (12mm) followed by *A. flavus* str1 (10 mm) and *A. clavatus* (10 mm) on CAS agar media (Graph 1). However the production of siderophore was found to be low in *A. terreus* (4.6 mm) and negligible in *A. fumigatus* both in agar cultures and in broth cultures. The amount of siderophore in broth was well accounted in case of *A. niger* (0.023) and *A. flavus* str2 (0.021) at 450nm. At 500nm the production of siderophore was significantly higher in *A. flavus* str1 (0.031) followed by *A. niger* (0.032), (Graph 2).

Graph1



Siderophore production by fungal isolates from the rhizosphere of P.nigrum L. on CAS solid media showing zone of production of siderophore

Graph 2

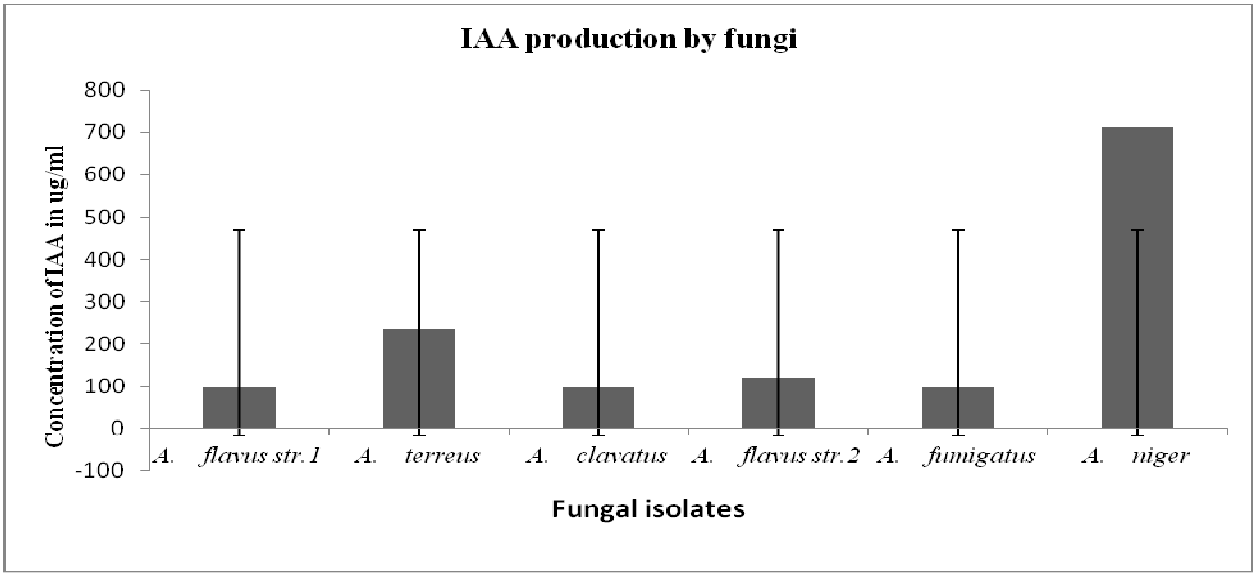


Siderophore production by fungal isolates in CAS broth media showing OD at 450nm and 500nm from the rhizosphere of P. nigrum L.

IAA production

Indole acetic acid production from tryptophan using broth cultures showed the greatest production in case of *A. niger* (Graph 3). 710 µg mL^{-1}) followed by *A. terreus* (235 µg mL^{-1}) isolated from the rhizosphere of pepper (The other isolates of *Aspergillus* species tested produced IAA in negligible quantities **–** *A. clavatus* (100 µg mL^{-1}), *A. flavus* str 1 (98 µg mL^{-1}), and *A. flavus* str2 (120 µg mL^{-1}).

Graph 3

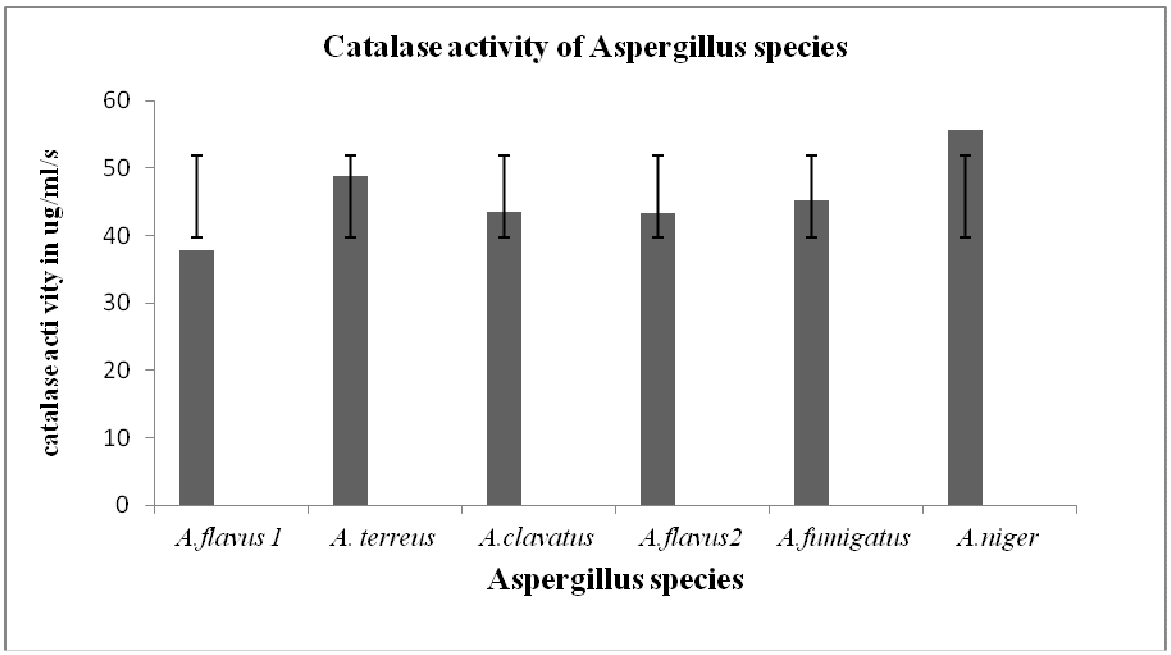


Indole acetic acid production by fungal isolates from the rhizosphere of P. nigrum L.

Catalase Production

All the fungal isolates of fungi showed good activity for the catalase enzyme since all the organisms are aerobic in nature**.** However the highest activity was detected in *A. niger* showing 55.8 ụg $mL^{-1}s^{-1.}$ The other organisms with comparatively good catalase activity were *A. flavus* str2 (43. 52 ụg $mL^{-1}s^{-1}$) and *A. terreus* (48.78 ụg $mL^{-1}s^{-1}$) (Graph 4).

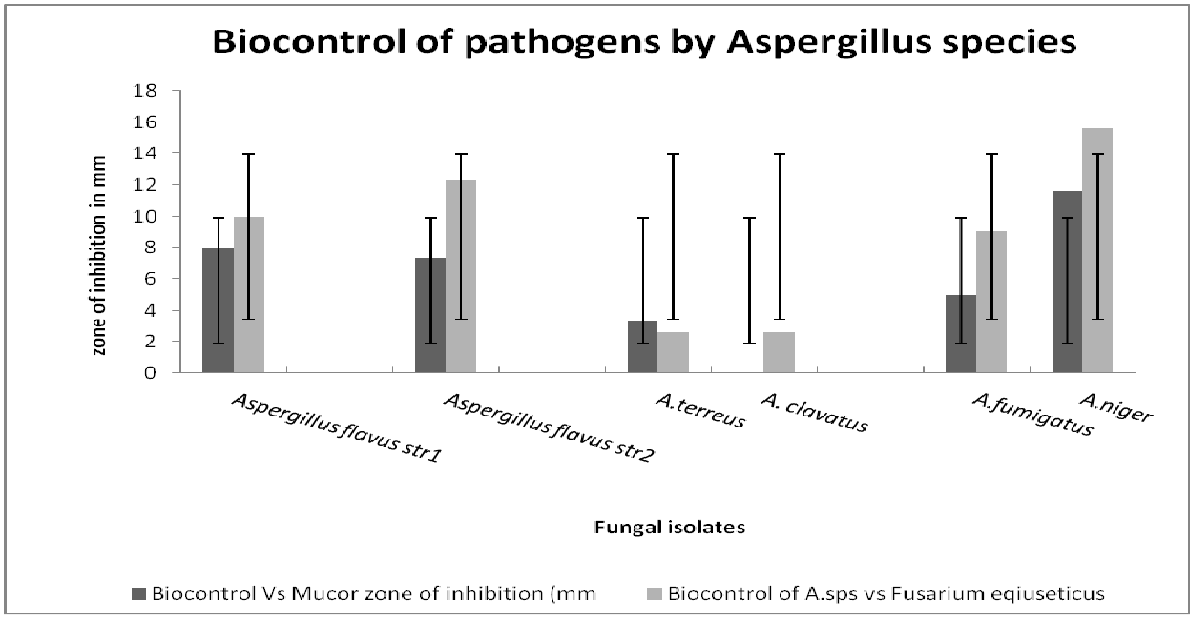
Graph 4



Catalase activity of fungal isolates from rhizosphere of P. nigrum L. Biocontrol of pathogens

It is evident from the observations made that antagonistic activity of *A. niger* is greater against *Fusarium equiseticus* (15.6 mm) than towards Mucor spp. (11.6 mm) (Graph 5). So also *A. niger* showed a better antagonistic effect towards both the organisms *Mucor spp*. And *Fusarium equiseticus* than any other *Aspergillus* species tested. The other species of *Aspergillus* with comparable activity against the pathogens are *A. flavus* str 2 showing 12.3mm inhibition zone against *F. equiseticus* and 7.3 mm zone towards *Mucor* spp. and *A. flavus* str 1 with a significant activity against *F. equiseticus* and a fairly moderate activity against *Mucor* spp.

Graph 5



Biocontrol of pathogens - Mucor sp.and Fusarium equiseticus by Aspergillus species on CDA media indicating zone of inhibition (mm) of growth by test organisms isolated from the rhizosphere of P. nigrum L.

DISCUSSIONS

The present experimental results shows that the six species of *Aspergillus* tested for various parameters have shown varied results with respect to the different characteristics of soil rhizospheric fungi. It is clearly seen that out of the six species, *A. niger* has been identified up to molecular level and has been identified as *A. niger* var. *niger* Tiegh. (1867) with similarity to *A.awamori* Nakaz. (1915) and *A. niger* var. *phoensis* (Corda) Al-Musallam (1980), [35]. It has been shown thoroughly well that it can be used as a PGPF organism, since it indicates higher production of siderophores and IAA production and has shown excellent activity for the catalase enzyme and a good potent phosphate solubilizer [36] along with good biocontrol activity towards the pathogens *F. equiseticus* and *Mucor sp*. *A.niger* and *A.flavus* have been reported to be excellent producers of IAA as seen in several other investigations[37]. Rhizospheric soil microbes are influenced by the plant roots through several mechanisms like root exudates, organic compounds, competition for nutrients, and the presence of surface for adhesion. Any microbial utilization for agriculture requires an evaluation of environmental factors along with introduction of indigenous organism in to the rhizosphere of

the crop plants and assessment of conditions suitable for effective and successful establishment of PGPR organism, [38, 26].

The production of siderophores by rhizospheric organisms is dependent on several factors like high iron concentrations in the soil as reported by Machua and Milagres, [12], for *A.niger* a non-typical siderophore producer which also reacted with CAS reagent. *A. niger* is known to be a good producer of citric and other organic acids which are thought to play a role in the reaction with CAS in presence of iron, [39]. Siderophore production by *A.niger* and *A. flavus* in both solid and broth media is well supported by studies by other workers. The findings of the present investigations have shown that IAA production by *Aspergillus* species could be explored further and exploited for agricultural use. However further studied are required in this direction to establish the exact contribution by *Aspergillus* species as PGPF organisms and as growth promoters, [40]. Several species of PGPF have been shown to trigger systemic resistance against various pathogens in cucumber plants[41]. Plant growth promoting fungi (PGPF), have been reported to be beneficial to several crop plants not only by promoting their growth but also by protecting them from diseases, [25]. In the present investigations it is clearly evident that organisms belonging to *Aspergillus* species have all the traits of PGPF and thus be potentially used as biofertilizers. They may be further investigated for their biocontrol activity against soil borne pathogens like *Fusarium, Mucor, Pythium*, etc. whose eradication by other conventional methods is difficult.

In the recent years eradication of soil borne pathogens like *Fusarium, Pythium*, etc has become increasingly difficult. The role of biocontrol agents (BCA) is becoming increasingly crucial and important in replacing the use of chemical fungicides where antagonistic fungi play a vital role in controlling the pathogens. Fungal based BCAs have gained wide acceptance next to bacteria primarily due to their broader spectrum I terms of disease control and yield, [25]. The present study has demonstrated that the ability of the different species of *Aspergillus* in the production of siderophores and bioactive compounds like IAA, in the production of catalase enzyme resulted in these organisms to inhibit the phytopathogens, *F.equiseticus* and *Mucor sp*. *A.niger* has been reported as a potential biocontrol agent due its ability to grow rapidly in soils compared to the pathogens thereby inhibiting them, the effective antagonist grew at a very fast rate out spacing the growth of the pathogen. Their interaction showed inhibition of growth of the pathogen by completion for the nutrients[42] .

CONCLUSION

The present investigations indicate that two out of the six fungal organisms isolated from the rhizosphere of the pepper plant have a good potential of being used as PGPF organisms as also biofertilizers. This group of fungi is poorly investigated in this regard and further study is required to establish their role in field areas. Although some work is done in the use of these fungi in the field of agriculture, their role mainly as plant growth promoters is yet to be exploited.

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