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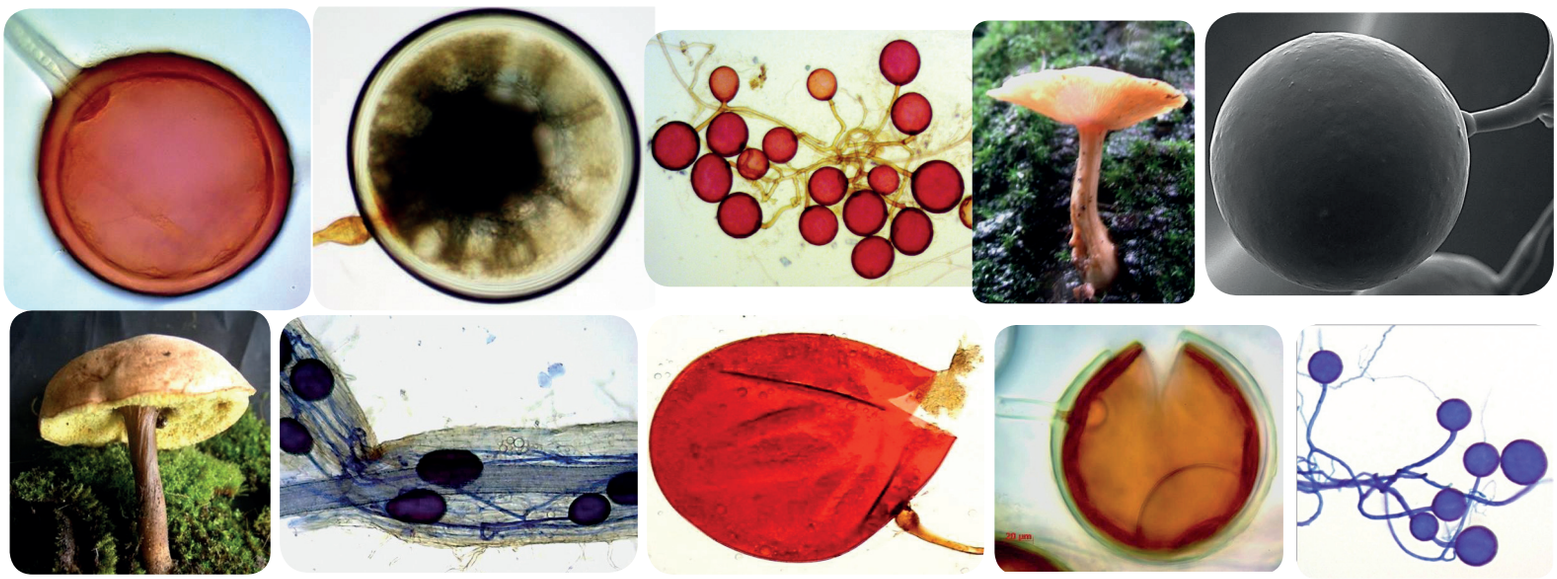

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Research finding paper

Induction of Systemic Resistance Through Activation of Host Defence Response in Safflower (*Carthamus tinctorius L.*) Against Fusarium Wilt by Arbuscular Mycorrhizal Fungi and Plant Growth-promoting Rhizobacteria

Amballa Hindumathi[1], Bhumi Narasimha Reddy[1], and Ammana Sabitha Rani[2]*

Abstract

Arbuscular mycorrhizal fungal (AMF) species, *Glomus fasciculatum*, PGPR strains *Pseudomonas putida* (Pp) and *Bacillus* sp. (Bac) were studied for their effect on plant growth parameters and induction of systemic resistance against *Fusarium oxysporum* f. sp. *carthami* (Path) in safflower (*Carthamus tinctorius* L.), both singly and in combination with or without pathogen. All the tested microorganisms showed a stimulatory effect in the form of significant increase in shoot and root length, plant (shoot+root) fresh and dry weight, and seed yield at two intervals of crop growth over control. Combined inoculation with AMF+Pp+Bac enhanced growth parameters, in addition to significant reduction in percentage disease incidence. AMF+Path treated plants recorded the least disease incidence (13.33%), followed by *P. putida* (17.00%) and *Bacillus* sp. (22.67%) when compared to only pathogen inoculated plants (75.33%). Disease incidence in co-inoculation with AMF+Pp+Bac+Path further reduced and turned out to be the least (8.67%). Increase in the activity of defence-related enzymes such as L-phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase were recorded in the individual bio-inoculant treatments in pathogen-infested soil. Even higher levels increase in the activity of defence-related enzymes were detected in the plants inoculated with the combination: AMF+Pp+Bac+Path. An increase in phenol content was also recorded in bio-inoculant (single)-treated plants and maximum levels were detected in combination inoculated plants. The tested bio-inoculants—*G. fasciculatum*, *P. putida*, and *Bacillus* sp.—showed promising results in reduction of disease incidence and were effective in inducing defence responses, and triggering resistance against Fusarium wilt pathogen (*Fusarium oxysporum* f. sp. *carthami*) in safflower.

Keywords

Arbuscular mycorrhizal fungi, defence-related enzymes, fusarium wilt, induced systemic resistance, plant growth-promoting rhizobacteria, safflower

Introduction

Plants are always found to be in symbiotic relationship with soil microbes (bacteria and fungi). The bacteria confer diverse beneficial effects to the host plant through various mechanisms such as nitrogen fixation, phosphate solubilization, growth hormone production, siderophores, environment pollutant degradation, antibiotic or lytic enzyme production and plant growth regulation. These bacteria are generally termed as plant growth-promoting rhizobacteria (PGPR) (Kloepper, Leong, Teintze, *et al*. 1980). The control of plant diseases using antagonistic bacteria

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is now being considered promising alternative to hazardous chemical fungicides. Several studies have demonstrated that the diverse rhizobacteria with biocontrol activity were able to reduce the use of agrochemicals (Adesemoye and Kloepper 2009). The PGPR are competitive plant root colonizers that belong to different genera and species. The most widely reported strains— *Pseudomonas* and *Bacillus* spp.—are the most studied rhizobacteria that are known to trigger induced systemic resistance (ISR) (Kloepper, Ryu, and Zhang 2004; Van Wees, Van der Ent, and Pieterse 2008). Studies on the mechanisms showed that PGPR promote plant growth directly by producing plant growth regulators or stimulating nutrient uptake and indirectly by producing siderophores and/or by producing antibiotics to protect plants from soil-borne phytopathogens or deleterious rhizobacteria. Studies on the mechanisms of biological control by PGPR revealed that some PGPR protect plants from several pathogens and pests by inducing systemic resistance (ISR) in them, activating defence mechanisms by triggering signalling pathways that lead to increased pathogen resistance.

The interactions of growth-promoting microbial population in the rhizosphere of arbuscular mycorrhizal plants have been studied by several scientists (Barea, Bonis, and Olivares 1983; Pacovsky and Fuller 1985; Linderman 1988). In addition, Gosling, Hodge, Goodlass, *et al*. (2006) reported that inoculation of plants with arbuscular mycorrhizal fungi (AMF) resulted in enhanced uptake of plant water and mineral nutrients. AMF have also been shown to scavenge the available phosphorus through their extraradical hyphae (Liu, Hamel, Hamilton, *et al*. 2000; Bianciotto and Bonfante 2002). The hyphae of these symbiotic AMF provide an increased area for the interaction of plants with other soil microorganisms in the mycorrhizosphere, thereby effecting root development as well as performance, and induce plant systemic resistance against various soil-borne root pathogens (Gosling, Hodge, Goodlass, *et al*. 2006). Filion, St-Arnaud, and Fortin (1999) reported that in transformed carrot roots the exudates produced by extraradical mycelium of *G. intraradices* significantly inhibited the conidial germination of the plant pathogen *Fusarium oxysporum*. *G. intraradices* has been shown to elicit induced systemic resistance against the parasitic nematodes—*Radopholus similis* and *Pratylenchus coffeae*—in banana plants (Elsen, Gervacio, Swennen, *et al*. 2008). Earlier studies showed that AM bioprotection is associated with stimulation of plant defence mechanisms (Baltruschat and Schönbeck 1972; Dehne and Schönbeck 1979). Previous results indicate that this mechanism is signalled by modulations such as lignification, induction of cell wall apposition (comprising callose) and accumulation of pathogen-related proteins or phenolic compounds (Pozo, Cordier, Dumas-Gaudot, *et al*. 2002; Dumas-Gaudot, Gollotte, Cordier, *et al*. 2000). Lignification in response to AM fungal colonization makes it difficult for the pathogenic hyphae to penetrate (Cordier, Gianinazzi, and Gianinazzi–Pearson 1996). Similarly, phenol accumulation in response to colonization by AMF was reported to cause both localized and systemic induced resistance to pathogens. The plants inoculated with AMF *Glomus versiforme* showed increased levels of soluble phenol contents in the roots of tomato which resulted in decrease in the population of *Ralstonia solanacearum* in the rhizosphere and the xylem tissues of the plant (Zhu and Yao 2004). Pozo, Cordier, Dumas-Gaudot, *et al*. (2002) observed that the AMF *G. mosseae* inoculation resulted in the reduction of *Phytophthora parasitica* infection in tomato roots by inducing mycorrhiza-related hydrolytic enzymes such as chitosanases and ß-1, 3 glucanase, that have lytic activity on the pathogen's cell wall. Thus, symbiotic microorganisms, for example, AMF and plant PGPR can induce systemic resistance against both air and soil-borne pathogens.

Safflower (*Carthamus tinctorius* L.) is one of the major oilseed crops in the world and is valued for its highly nutritious edible oil that has high-commercial value. India ranks first in terms of area used for production of safflower (54%) and contributes nearly 40% production in the world. Diseases are one of the major constraints in safflower cultivation that cause heavy losses to the farmers. Amongst the fungal diseases, Fusarium caused by *Fusarium oxysporum* f. sp. *carthami*, is one of the major diseases responsible for a substantial loss in quality and yield of the crop (up to 55%–65% at different stages of the crop). Factors, for instance, the wide host range of the pathogens, their ability to survive under arid conditions, and expensive and ineffective fungicides have made scientists and farmers search for a cost-effective alternative to control soil-borne phytopathogens. In plants exhibiting ISR, a number of reactions have been observed such as deposition of callose, lignin, and phenolics beyond infection sites, increase in activities of chitinase, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) (Chen, Belanger, and Benhaou 2000), lipoxynase and 1, 3 glucanase, enhanced phytoalexin production (Van Peer, Niemann, and Schippers 1991), and induced expression of stress-related genes.

In the light of the mentioned events, an attempt was made to determine the increase in the activity of enzymes involved in induced resistance: PPO, PAL, and the phenol content. In safflower, reports on the induction of systemic resistance using beneficial

microorganisms are scarce. Therefore, the present study was aimed to test and identify the potential indigenous AMF and native PGPR isolates from safflower rhizosphere soil as biological control agents, trigger ISR against Fusarium wilt pathogen, *Fusarium oxysporum* f. sp. *carthami* and their ability to boost plant defence mechanisms.

Materials and Methods

The rhizosphere soils of safflower, *Carthamus tinctorius* L., agriculture fields in different localities of Telangana, India were screened for PGPR and AMF species using standard techniques and specific media.

Isolation of native strains of plant growth-promoting rhizobacteria isolates

Rhizobacteria were isolated by dilution plate technique using specific media, and prominent colonies were selected, purified, maintained on nutrient agar slants and stored at 4°C until further use.

Isolation and maintenance of pathogenic fungi

The Fusarium wilt-causing plant pathogenic fungus—*Fusarium oxysporum* f sp. *carthami*—was isolated, using potato dextrose agar medium, from the diseased safflower plant parts. The fungal pathogen was identified and confirmed by conducting pathogenicity test on the safflower plants, and then stored at 4°C for experimental use.

Characterization and identification of rhizobacteria

The selected rhizobacteria were characterized through morphological, biochemical, and physiological tests according to *Microbiology: a laboratory manual* (Cappuccino and Sherman 2004).

Molecular identification of the selected rhizobacterial isolates

The selected rhizobacterial isolates were sent to Macrogen Inc., Seoul, Korea, for identification by 16S rDNA gene sequencing analysis. The 16S rDNA sequences were further compared with the available reference nucleotide sequences from the database of National Center for Biotechnology Information (NCBI) and European Molecular Biology Laboratory (EMBL), and the accession numbers obtained. Phylogenetic trees were constructed for the isolates by retrieving the bacterial strain names having highest scores of percentage similarity and using Neighbour-joining method (Saitou and Nei 1987).

Mass multiplication of native plant growth-promoting rhizobacteria isolates

The inoculum of *Pseudomona putida* and *Bacillus* sp. (selected for seed bacterization) was multiplied by growing the isolates in nutrient broth on a rotary shaker at 180 rpm at 28 ± 2°C for 36 h. The inoculum load was adjusted spectrophotometrically to 0.45 optical density (OD) at 610 nm wavelength to obtain a concentration of 1×10^8 cfu/mL (cfu: colony-forming units).

Mass multiplication of the arbuscular mycorrhizal fungi *Glomus fasciculatum*

Individual AMF spores were isolated using wet sieving and decanting method (Gerdemann and Nicolson 1963) from air-dried safflower rhizosphere soil samples. The AMF spores were identified using standard key (Schenck and Perez 1990; Wu, Hao, and Lin 2002) and by comparing with the morphological description of spores presented in the reference culture information[3] of INVAM 2013 (Hindumathi and Reddy 2016). The predominantly occurring AMF species, *G. fasciculatum* (Hindumathi and Reddy 2015), was cultured in a sterilized substrate sand and soil mixture (1:1), and grown for 4 months with sorghum as host. The substrate containing the mixture of spores, mycorrhizal root segments and hyphae served as the stock culture of AMF inoculum.

Plant material

One of the popular safflower seed varieties, Manjira, was obtained from the Safflower Research Station, Department of Agriculture, Tandore, Telangana. The surface sterilized seeds were treated with the rhizobacteria (1×10^8 cfu/mL bacterial suspension) using 1% carboxy methyl cellulose (CMC) as adhesive, and shade-dried overnight aseptically under a laminar air-flow. The seed soaked in sterilized distilled H_2O amended with CMC was used as control.

Test pathogen (*Fusarium oxysporum* f. sp. *carthami*) inoculum preparation

The inoculum was prepared by culturing the fungus on potato dextrose agar plates for 7 days at 28 ± 2°C. The conidial suspension was prepared by adding 20 mL of sterilized distilled H_2O to each plate and then adjusting it to 1000 conidia mL^{-1}.

Pot experiment

The study was conducted using an efficient AMF species, *G. fasciculatum*, and two potential native rhizobacterial isolates that exhibited plant growth-promoting ability and efficient antimicrobial activity *in vitro*. They were molecularly characterized as *Bacillus* sp. (deposited at NCBI GenBank with strain accession No. KT986296) and *Pseudomonas putida* (Accession No. KT986299) and then tested for plant growth promotion, biological suppression of disease incidence and induction of systemic resistance.

[3] Details available at http://invam.caf.wvu.edu/fungi/taxonomy/species/D.htm

The different microbial treatments used, individual and in combination with or without the pathogen, for inducing systemic resistance included: 1-control (natural field soil), 2-*G. fasciculatum* (AMF), 3-*Pseudomonas putida* (Pp), 4-*Bacillus* sp. (Bac), 5-test pathogen *Fusarium oxysporum* f. sp. *carthami* (Path), 2+5, 3+5, 4+5, 2+3+4, and 2+3+4+5. The field soil was autoclaved three times (24 h interval between each sterilization cycle: 20 min. at 120°C). The pathogen and bacterial inoculations were done by following standard protocols. Mycorrhizal inoculum of *G. fasciculatum* (containing 20 spores/g and root segments with 70% colonization) was placed in a layer, 2 cm below the seed hole. Bacterized and untreated seeds (10 seeds) were sown in each pot (4 kg) with or without the pathogen. Thinning, down to 5 seedlings/ pot, was done on the 12th day after emergence. The pots were arranged randomly, maintaining three replicates for each treatment. Hoagland nutrient solution (Hoagland and Arnon 1938), without phosphorus, was added to the plants inoculated with AMF, at regular intervals. Observations of growth parameters, shoot and root lengths (cm), their fresh and dry weights (g/plant), dried at 70°C for 72 h to a constant weight. The observations were recorded at two intervals of growth period, that is, 30 and 60 days after sowing (DAS). Grain yield (g) per plant and the number of diseased plants were recorded at the time of harvest. Disease incidence (%) was calculated using the formula:

$$\text{Disease incidence (\%)} = \frac{\text{No. of plants showing disease symptoms}}{\text{Total number of plants observed}} \text{ X } 100$$

Assay of defence-related enzymes in host plant

To study induction of systemic resistance, defence-related enzymes—L-phenylalanine ammonia lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO)—in the control and inoculated host plants, were assayed. Enzyme assay was done every 15 days, from the 15th day till the 90th day after sowing (DAS). The phenol content was also assayed. Leaf samples of safflower plants from treated and control plants were collected in every 15 days, (from the 15th day till the 90th day) and immediately stored in ice-boxes. Their extract was assayed to determine the aforementioned enzymes' activities by following standard procedures.

Determination of L-phenylalanine ammonia lyase activity

The reaction mixture consisted of 1 mL of the enzyme extract, 0.5 mL of 0.2 M borate buffer (pH 8.7), 1.3 mL of distilled H_2O, and 0.2 mL of 1 M L-phenylalanine. Changes in absorbance at 290 nm were noted. Reaction mixture without the substrate was considered as control. One unit of enzyme activity produced 3.37 mM of cinnamic acid/ hour (Singh and Prithviraj 1997). Three replicates were maintained for each treatment.

Assay of polyphenol oxidase activity

The polyphenol oxidase (PPO) activity was assayed according to Mayer, Harel, and Shaul (1965). 2 mL of the enzyme extract and 3 mL of phosphate buffer (pH 6) were taken in a cuvette tube and the absorbance was adjusted to 'zero' at 495 nm. To this 1 mL of 0.05 M guaiacol was added and mixed thoroughly. The changes in OD were noted every 30 s. for 3 min., and the change in 0.01 OD was taken as one unit of enzyme activity. The reaction mixture with heat-inactivated enzyme served as blank. The mean change in absorbance was calculated for 1 min. and the activity was expressed in units/gram of fresh weight.

Assay of peroxidase activity

1.5 mL of 20% H_2O_2, 1.5 mL of phosphate buffer (pH 6), and 1.5 mL of 0.05 M guaiacol were taken in a cuvette tube and the absorbance was adjusted to 'zero' at 470 nm. To this 1 mL of the enzyme extract was added and mixed thoroughly. The changes in OD was noted every 20 s for 3 min., and the average change in absorbance between 30 s and 180 s was taken to plot POX activity which was expressed in units/gram of fresh weight.

Estimation of phenol content

1 g of fresh sample was homogenized with 10 mL of 80% methanol and the volume adjusted to 15 mL at 70°C (Zieslin and Ben-Zeken 1993). 1 mL of methanolic extract was added to 5 mL of distilled H_2O and 250 µL of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25°C. The absorbance of the blue colour mixture was read at 725 nm. Catechol was used as standard.

Statistical analysis

The data was statistically analysed by factorial analysis of variance (ANOVA) using WINDOSTAT software, Version 9.2. The mean values were compared using the least significant difference (LSD) test ($p \leq 0.001$) to detect significant differences among the treatments. The values of percentage disease incidence were arcsine transformed before statistical analysis.

Results and Discussion

Isolation and identification of pathogen

The pathogen was identified based on morphological, conidia, and culture characteristics using standard manuals and confirmed through a pathogenicity test.

Physiological and biochemical traits of the selected growth-promoting rhizobacteria isolates

Two isolates that showed large inhibition zones, against the test pathogen *Fusarium oxysporum* f. sp. *carthami*, were selected for the study. The two putative PGPR strains (numbered OUHS05 and OUHS02), on analysis of 16S rRNA sequences by neighbour-joining method, matched *Pseudomonas putida* strain DLL-E4 (100% homology) and *Bacillus* sp. strain YB-1401 (100% homology). The 16S rRNA nucleotide sequences of the strains OUHS05 and OUHS02 were submitted to NCBI GenBank and accession numbers of the strains were obtained as KT986299 and KT986296, respectively. Morphological and biochemical characterization also supported the identification process.

Effect of *Glomus fasciculatum, Pseudomonas putida*, and *Bacillus* sp. strains on safflower

The results of individual and co-inoculation of *Carthamus tinctorius* L. with *G. fasciculatum* (AMF), *Pseudomonas putida* KT986299 (Pp), and *Bacillus* sp. KT986296 (Bac) strains, in the presence or absence of the pathogen, are noted on plant growth parameters at 30 and 60 days. The parameters noted in terms of shoot and root lengths, and fresh and their dry weights are presented in Tables 1 and 2, respectively.

The data showed that the three individual treatments had a stimulatory effect on safflower plants and significantly increased the shoot and root lengths, plant (shoot+root) fresh and their dry weights when compared to control and pathogen-inoculated plants. The plants with AMF+Pp+Bac inoculation exhibited enhanced growth parameters, resulting in a 2-fold increase in shoot and root lengths over control in growth period of both 30 and 60 days. A similar trend was observed in bio-inoculant treated plants in the presence of the pathogen when compared to control and only pathogen-inoculated plants.

In contrast, the plants co-inoculated with the three bio-inoculants showed a 3-fold increase in fresh and their dry weights over control plants at 30 days, however, a 2-fold increase was observed at 60 days. The plants treated with the combinations, in the presence of the pathogen, showed a 3-fold increase in fresh weights (30 days) and dry weights (60 days and 4-fold increase in 30 days crop when compared to only pathogen-inoculated plants. Similar results were observed in treatments involving the bio-inoculants and the pathogen over only pathogen-treated plants and the results are on par with rest of the treatments.

Table 1 Influence of AMF and PGPR strains on shoot and root lengths of inoculated safflower

Treatments	Shoot length		Root length	
	30 days	60 days	30 days	60 days
Control	22.580^{f}	33.450^{f}	9.140^{fg}	16.740^{d}
G. fasciculatum	37.350^{bc}	52.290^{b}	15.720^{bc}	22.640^{ab}
Pseudomonas putida	38.480^{bc}	49.450^{bc}	16.840^{ab}	20.570^{bcd}
Bacillus sp.	34.720^{cd}	45.750^{cd}	14.720^{bcd}	20.650^{bcd}
Gf + Pp + Bac	43.640^{a}	58.640^{a}	20.480^{a}	25.730^{a}
Pathogen	12.090^{g}	26.260^{g}	7.450^{g}	11.690^{e}
G. fasciculatum + path	34.823^{bcd}	45.280^{cde}	12.740^{cdef}	19.690^{bcd}
Pp + path	31.737^{de}	42.267^{de}	11.510^{def}	19.450^{bcd}
Bac + path	28.420^{e}	40.840^{e}	10.750^{efg}	17.680^{cd}
Gf + Pp + Bac + path	39.460^{ab}	52.730^{b}	13.797^{bcde}	21.390^{bc}
Gen. Mean	**32.330*****	**44.696*****	**13.315*****	**19.623*****
F Prob.	0	0	0	0
SEM	1.585	1.574	1.273	1.353
CD 1%	6.452	6.408	5.181	5.509

Note: Values followed by different superscripts in each column were significantly different at $p \leq 0.001$.

Among the three isolates used as individual treatments, AMF inoculation resulted in the highest plant growth parameters, followed by *P. putida* and *Bacillus* sp. Combined inoculation with AMF+Pp+Bac produced maximum growth at both the growth periods. The growth parameters of the pathogen-inoculated plants were found to be the least.

Seed yield

The effects of individual and co-inoculation of safflower with AMF, Pp and Bac, with or without pathogen, on seed yield have been presented in Table 2. Seed yield was found to be 2.1 times as that of control in the plants inoculated with AMF+Pp+Bac and 3.5 times as that of the pathogen-inoculated plants in the AMF+Pp+Bac+Path treatment. This was significantly

Table 2 Influence of AMF and PGPR strains on fresh weight, dry weight (shoot+root), and seed yield of safflower

Treatments	Fresh weight (g/plant)		Dry weight (g/plant)		Seed yield (g/plant)
	30 days	60 days	30 days	60 days	
Control	7.748^{ef}	52.641^{e}	1.816^{bc}	14.174^{e}	1.830^{g}
Glomus fasciculatum	14.264^{bc}	88.972^{bc}	3.019^{ab}	23.712^{bc}	3.593^{b}
Pseudomonas putida	13.374^{bcd}	86.495^{bcd}	2.984^{ab}	20.850^{cd}	3.282^{c}
Bacillus sp.	10.722^{bcde}	82.149^{cd}	2.234^{bc}	18.749^{de}	3.045^{e}
Gf + Pp + Bac	20.142^{a}	109.748^{a}	4.283^{a}	30.162^{a}	3.925^{a}
Fusarium oxysporum (path)	5.654^{f}	32.725^{f}	0.894^{c}	8.269^{f}	1.072^{h}
G. fasciculatum + path	12.714^{bcd}	80.697^{d}	2.648^{b}	15.296^{e}	3.160^{d}
Pp + path	10.262^{cde}	82.831^{cd}	2.472^{b}	16.489^{de}	2.954^{ef}
Bac + path	9.721^{def}	80.042^{d}	2.014^{bc}	15.348^{e}	2.883^{f}
Gf + Pp + Bac + path	14.672^{b}	92.326^{b}	3.018^{ab}	26.749^{ab}	3.785^{a}
Gen. Mean	**11.927** ***	**78.863****	**2.538****	**18.980****	**2.861****
F Prob.	0	0	0.007	0	0
SEM	1.427	2.730	0.460	1.668	0.031
CD 1%	5.808	11.114	1.871	6.790	0.127

Note: Values followed by different superscripts in each column were significantly different at $p \leq 0.001$.

G. fasciculatum, *P. putida* (KT986299), and *Bacillus* sp. (KT986296) strains had a positive effect on plant growth promotion even though they differed in their relative effects on growth.

Inoculation with AMF+Pp+Bac further enhanced the growth of the host plants, in agreement with the previous results where the growth and biomass yield of *Ocimum basilicum* improved on inoculation with *G. fasciculatum* and PGPR (Hemavathi, Sivakumar, Suresh, *et al*. 2006). Plant growth promotion by *G. fasciculatum*, *P. putida,* and *Bacillus* sp. is usually attributed to factors such as maximized nutrient and water uptake (by AMF), increased nutrient availability, and production of phytohormones by PGPR.

higher over control plants. Among the individual inoculations, *G. fasciculatum* caused maximum seed yield (2 folds), followed by *P. putida* and *Bacillus* sp. 1.8 and 1.6 times, respectively over the control. Similar trend was observed in the plants treated with bio-inoculants in the presence of the pathogen. Overall results showed that *G. fasciculatum* caused maximum seed yield, followed by *P. putida* and *Bacillus* sp, resulting in nearly 3-folds, 2.8, and 2.7 times, respectively over pathogen-treated plants.

Disease incidence

The data presented in Table 3 shows a significant reduction in the percentage of disease incidence in the plants treated with the three bio-inoculants, in

the presence of the pathogen (Path) *F. oxysporum* f. sp. *carthami*. Plants inoculated with AMF+Path recorded lowest percentage disease incidence (13.33%), followed by *P. putida* (17%), and finally *Bacillus* sp. (22.67%), compared to only pathogen- inoculated plants (75.33%). The disease incidence in the combined inoculation with AMF+Pp+Bac+Path was found to be even less (producing the least- 8.67%). Among the bio inoculants, *G. fasciculatum* showed greater reduction in disease severity (82.33%), followed by *P. putida* (77.43%) and *Bacillus* sp. (69.91%), in addition to increase in plant growth promotion. Furthermore, maximum reduction (91.33%) in disease severity was recorded in plants co-inoculated with AMF+Pp+Bac+Path.

Effect of microbial treatments on defence-related enzymes' activity in the host

- L-PAL activity

The effect of different bio-inoculants, in the presence of the pathogen, on the enzyme activity significantly increased till 90 days (Table 4). The highest activity of PAL was recorded in the plants inoculated with AMF+Pp+Bac, grown in the pathogen-infested soil till 90 days. The pathogen-treated plants showed sharp and gradual increase in PAL activity from 15 to 45 days after which the activity decreased gradually and finally showed no activity after completion of 90 days. Throughout the study, the lowest enzyme activity was observed in control plants.

Table 3 Influence of AMF and PGPR on disease incidence in and phenol content of infected safflower

Treatments	Disease incidence (%)	Phenol content (mg/g)	DI (%) (Arc Sin)
Control	0.000^{f}	1.907^{d}	4.055^{f}
Pathogen	75.333^{a}	1.123^{e}	60.238^{a}
G.fasciculatum + path	13.333^{d}	3.833^{b}	21.404^{d}
Pp + path	17.000^{c}	3.933^{b}	24.333^{c}
Bac + path	22.667^{b}	3.533^{c}	28.426^{b}
Gf + Pp + Bac + path	8.667^{e}	4.633^{a}	17.098^{e}
Gen. Mean	**22.833** ***	**3.161** ***	**25.926** ***
F Prob.	0	0	0
SEM	0.823	0.042	0.625
CD 1%	3.690	0.189	2.803

Note: Values followed by different superscripts in each column were significantly different at $p \leq 0.001$.

Table 4 Influence of AMF and PGPR on L- PAL activity of infected safflower

Treatments	15 days	30 days	45 days	60 days	75 days	90 days
Control	14.000^{d}	14.000^{e}	16.000^{f}	16.000^{f}	18.333^{f}	17.000^{e}
Pathogen	25.000^{c}	34.000^{d}	52.000^{e}	32.000^{e}	22.000^{e}	0.000^{f}
Gf + path	38.000^{b}	54.000^{b}	78.000^{b}	92.000^{b}	126.000^{b}	138.000^{b}
Pp + path	34.000^{b}	51.000^{bc}	72.000^{c}	86.000^{c}	108.000^{c}	121.000^{c}
Bac + path	32.000^{b}	49.000^{c}	64.000d	78.667^{d}	96.000^{d}	108.000^{d}
Gf + Pp + Bac + path	46.000^{a}	68.000^{a}	102.000^{a}	126.000^{a}	142.000^{a}	154.000^{a}
Gen. Mean	**31.500** ***	**45.000** ***	**64.000** ***	**71.778** ***	**85.389** ***	**89.667** ***
F Prob.	0	0	0	0	0	0
SEM	2.119	1.300	1.234	1.009	0.524	0.810
CD 1%	9.496	5.825	5.530	4.523	2.346	3.629

Note: Values followed by different superscripts in each column were significantly different at $p \leq 0.001$.

- POX activity

The POX activity in different bio-inoculant (individual and combined)-treated plants in the pathogen-infested soil was significantly high compared to control and only pathogen-treated plants (Table 5). In other words, plants treated with the bio-inoculants showed relatively higher POX activity at all days of plant growth over control and only pathogen-treated plants. Furthermore, enzyme activity increased with increase in age. The enzyme activity was recorded maximum in AMF+Pp+Bac-treated plants. Lowest activity was observed in control plants throughout the study, whereas only pathogen-treated plants showed increasing activity to 45 days which decreased later, resulting in no activity at 90 days.

- PPO activity

The influence of AMF and PGPR on PPO activity of infected safflower is given in Table 6. Maximum activity was recorded in combined inoculation with AMF+Pp+Bac, throughout the plant growth period. The pathogen-treated plants showed increase in the enzyme activity up to 30 days and then a gradual decrease up to 60 days after which no activity was observed in 75 and 90 day plants. In control plants, the enzyme activity was found to be low up to 45 days, slightly increased after 60 and 75 days and decreased slightly after 90 days.

Table 5 Influence of AMF and PGPR on peroxidase activity of infected safflower

Treatments	15 days	30 days	45 days	60 days	75 days	90 days
Control	11.333^{f}	11.333^{e}	11.333^{f}	11.667^{f}	14.333^{f}	14.333^{e}
Pathogen	15.000^{e}	18.333^{d}	27.333^{e}	27.667^{e}	31.667^{e}	0.000^{f}
Gf+path	27.000^{b}	32.667^{b}	45.000^{b}	59.000^{b}	63.333^{b}	72.333^{b}
Pp+path	25.000^{c}	32.333^{b}	36.333^{c}	51.000^{c}	57.667^{c}	65.000^{c}
Bac+path	20.667^{d}	28.000^{c}	32.333^{d}	48.333^{d}	54.333^{d}	54.333^{d}
Gf + Pp + Bac + path	32.333^{a}	47.000^{a}	52.333^{a}	68.333^{a}	74.000^{a}	88.667^{a}
Gen. Mean	**21.889** ***	**28.278** ***	**34.111** ***	**44.333** ***	**49.222** ***	**49.111** ***
F Prob.	0.000	0.000	0.000	0.000	0.000	0.000
SEM	0.455	0.360	0.443	0.333	0.404	0.375
CD 1%	2.041	1.614	1.986	1.494	1.809	1.681

Note: Values followed by different superscripts in each column were significantly different at $p \leq 0.001$.

Table 6 Influence of AMF and PGPR on polyphenol oxidase activity of infected safflower

Treatments	15 days	30 days	45 days	60 days	75 days	90 days
Control	21.333^{e}	28.333^{f}	34.333^{e}	38.333^{e}	42.667^{e}	42.000^{e}
Pathogen	27.333^{b}	44.000^{e}	38.333^{d}	32.333^{f}	0.000^{f}	0.000^{f}
Gf + path	26.333^{c}	59.333^{b}	68.333^{b}	83.333^{b}	104.667^{b}	121.333^{b}
Pp+path	22.333^{d}	54.667^{c}	67.667^{b}	79.333^{c}	92.667^{c}	116.667^{c}
Bac+path	21.333^{e}	51.667^{d}	62.333^{c}	78.000^{d}	87.333^{d}	89.000^{d}
Gf+Pp+Bac+path	39.000^{a}	66.333^{a}	74.333^{a}	114.667^{a}	148.333^{a}	158.667^{a}
Gen. Mean	**26.278** ***	**50.722** ***	**57.556** ***	**71.000** ***	**79.278** ***	**87.944** ***
F Prob.	0.000	0.000	0.000	0.000	0.000	0.000
SEM	0.292	0.417	0.502	0.408	0.491	0.390
CD 1%	1.308	1.870	2.249	1.830	2.199	1.747

Note: Values followed by different superscripts in each column were significantly different at $p \leq 0.001$.

- Phenol content

Plants treated individually (with AMF, Pp or Bac) in the presence of the pathogen showed high accumulation of phenol compared to control and only pathogen inoculated plants (Table 3). In plants inoculated with AMF+Pp+Bac, the phenol content further increased to be the highest, while only pathogen-inoculated plants showed the least.

Induced systemic resistance against plant pathogens is mediated by effective PGPRs such as *Pseudomonas* spp. (Leeman, den Ouden, Pelt, *et al*. 1996) and *Bacillus* spp. (Kloepper, Ryu, and Zhang 2004). In majority of the investigations conducted so far, several strains of *P. fluorescens* and *Bacillus subtilis* have been reported to mediate elicitation of ISR in plants, resulting in significant reductions in the incidence or severity of various diseases. The PGPR-treated tomato seeds when challenge inoculated with bacterial and fungal pathogens showed increase in PAL activity and total phenol content (Ramamoorthy, Raguchander, and Samiyappan 2002). Similarly, induction of systemic resistance in tomato seeds treated with rhizobacteria against *P. syringae* pv. *tomato* and *Xanthomonas vesicatoria* and other fungal pathogens correlated with increased levels of PAL and POX in the plants (Silva, de Silva, Macagnan, *et al*. 2004). Zdor and Anderson (1992) observed that rhizosphere colonization of various bacteria induced PPO activity in bean. Higher PPO activity was recorded against *P. aphanidermatum* in cucumber roots inoculated with *Pseudomonas corrugata* (Chen, Belanger, and Benhaou 2000) and in chilli seedlings treated with PGPR (*Bacillus* spp. and *Pseudomonas* spp.) challenged with similar pathogen (Kavitha, Mathiyazhagan, and Senthilvel 2005). Peroxidase activity, associated with disease pathogenesis, leads to the strengthening of cell walls with the accumulation of phenolic compounds (Do, Hong, Jung, *et al*. 2003). Accumulation of phenolic compounds in response to infection leads to induced resistance (Hammerbacher, Ralph, Bohlmann, *et al*. 2011). Phenol and phenolic compounds have been reported to be fungitoxic in nature and increase the mechanical strength of the host cell wall by making it tougher and thicker and this helps to resist pathogen penetration and prevents degradation of plant cell wall.

The present study results showed increased accumulation of phenolic substances in response to infection by the pathogen. Based on the data which is in agreement with earlier studies on other plant species, colonization by both AMF and PGPR provides safflower with optimal growth conditions. Plants are capable of signalling soil microbes so as to facilitate the establishment of beneficial microbial community in the rhizosphere. Strigolactones produced by AMF induce branching in mycorrhizal hyphae, thus enhancing root colonization. Likewise, plants can produce chemical signals that elicit behavioural response in PGPRs. It was evidently seen that benzoxazinoid production was enhanced by mycorrhizal infection in the roots treated with *P. putida*. The isolates — *G. fasciculatum*, *P. putida,* and *Bacillus* sp. — used in the study were very effective in inducing the plants to synthesize higher levels of defence-related enzymes (PAL, POX, PPO) and accumulate phenol under greenhouse conditions and these results are in agreement with the results of Ramamoorthy, Raguchander, and Samiyappan (2002). Beneficial microbes such as AMF and PGPR are known for inducing systemic resistance against a broad spectrum of plant diseases by different mechanisms including, defence-related enzymes (Jogaiah, Abdelrahman, Tran, *et al*. 2018). In conclusion, both AMF and PGPR can act together on plant growth, presumably due to complementary impacts on soil nutrient solubilization and uptake. Moreover, co-colonization by AMF and PGPR seems to have strong synergistic effects on priming the host immunity.

The results of this study clearly indicate that the tested *G. fasciculatum*, *P. putida,* and *Bacillus* sp. resulted in suppression of disease incidence and hence are promising in inducing and/or priming defence responses and triggering resistance against the Fusarium wilt pathogen, *Fusarium oxysporum* f. sp. *carthami*, in safflower. Results also demonstrate that an appropriate combination of AMF and PGPR can significantly increase plant growth and seed yield, and elicit ISR. The exploitation of rhizosphere microorganisms for increasing the yield and crop protection seems to be an attractive approach in sustainable agriculture.

Conclusion

The application of dual inoculation of AMF and PGPR seems to be an effective treatment combination to improve plant growth parameters, increase yield, suppress disease incidence, and elicit ISR against the pathogens. The results also demonstrate the possibility of replacing chemical fertilizers with bio-fertilizers which in turn would help the small and marginal farming sectors in the sustainable production of safflower.

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News Highlights

Towards creating better crops

The discovery of a specific gene (that controls an important symbiotic relationship between plants and soil fungi) by a team of scientists led by the Department of Energy's Oak Ridge National Laboratory, USA, could lead to the development of bioenergy and food crops that can withstand harsh growing conditions, resist pathogens and pests, require less chemical fertilizer, and produce large and plentiful plants per acre.[1]

First key step in setting yield potential

With the interest of improving phosphate availability, Bayer Crop Science has come up with new biostimulant seed treatments—Jump Start WT and Pro Stablish WT. Applying them in combination effectively immensely increases the yield by making more phosphates available for seedling growth and stimulates the growth of mycorrhizal fungi which ensures phosphate availability in the immediate root zone/ rhizosphere. [2]

The ghost orchid

Dendrophylax lindenii (the ghost orchid), an endangered species limited to scattered populations in Cuba, the Bahamas and Florida, has long enchanted researchers whose current interest is to cultivate them and reintroduce them into the wild. The key is not only recreating precise conditions that they need to thrive, but also providing them with the right strain of a specific mycorrhizal fungus without which the seeds cannot germinate. [3]

[1] Details available at www.sciencedaily.com

[2] Details available at cropscience.bayer.co.uk

[3] Details available at www.mnn.com

For the Monarch

Major culprits responsible for the decline in the number of Monarch butterflies are climate change, pesticides and herbicides, deforestation in the mountains of Mexico and a dwindling supply of milkweed. Lack of knowledge on the specifics of the native milkweeds had been posing a major challenge in the native milkweed population until recently when Barbara Keller-Willy, founder of a non-profit organization, called Monarch Gateway, found evidence of a symbiont crucial to the plant's growth. [4]

Functional plant genes to reduce pollution

Researchers from the Boyce Thompson Institute, Cornell University, USA, have uncovered the function of a pair of plant genes that could help improve phosphate capture. The two genes are present throughout the plant kingdom. Being able to control these would mean controlling fungal colonization levels in plant roots as these genes have been found to be key modulators of AM fungal symbiosis. [5]

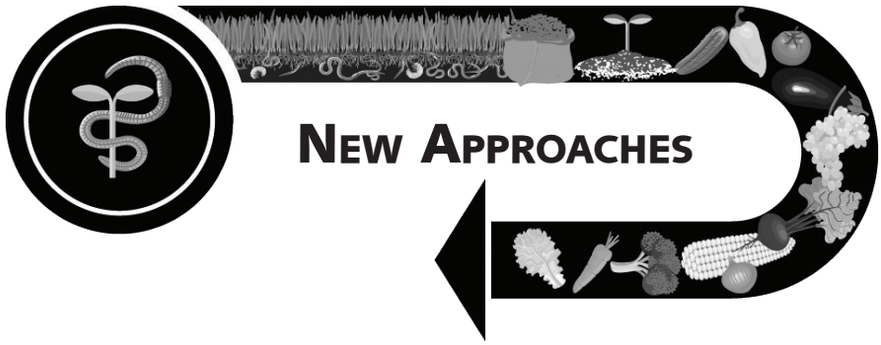
Using mycorrhizae pathway

Gyaneshwar Prasad, a UWM microbiologist from India, has created a biological model that allows to probe deeper into the communication pathways that rhizobia might use in rice. Prasad's research centres on finding a way to feed the world in a sustainable fashion. To do so, he's following a path already laid out by nature. [6]

[4] Details available at www.texasobserver.org

[5] Details available at www.sciencedaily.com

[6] Details available at www.newswise.com



New Approaches and Techniques

Mycothalli as Arbuscular Mycorrhizal Fungal Propagules

The mutualistic relationship established by mycorrhizal fungi has a substantial impact on the nutrition, growth, and productivity of host plants. For the isolation, taxonomy and preparation of arbuscular mycorrhizal fungal (AMF) inoculum, spores and root fragments have been used as potent sources. Generally, root trap culture has been used for isolating a single regenerated spore in order to establish a monospecific, native AMF line. It has been shown that up to three AMF isolates of different morphotypes could be cultivated monoxenically from 4–5 mm long root fragments. Although roots could be co-colonized by multiple AMF species, only a small portion of spores germinate and do so only under certain conditions. However, the co-existence of diverse species, proved by rDNA analysis, indicates that the majority of AMF colonize roots in a vegetative manner and that their functionality cannot be assessed by inoculation studies conducted in a spore-dependent manner. Thus, it would be beneficial to generate a vegetative, spore-independent, less space occupying, easy-to-cultivate (within a short period) and easy-to-maintain AMF inoculum for the assessment and deeper understanding of the diversity of AMF species and their functionalities. Considering that roots inevitably host numerous AMF species, a smaller size (that is, a lower cell number for colonization) may be more suitable for the isolation of a single AMF (Kobae, Ohtomo, Morimoto, *et al*. 2019).

This near-ubiquitous plant-fungal symbiosis, thought to have emerged more than 410 million years ago, is considered an integral part of land plant evolution (Field, Bidartondo, Rimington, *et al*., 2019; Kobae, Ohtomo, Morimoto, *et al*. 2019). The earliest divergent extant branches of the land plant phylogeny supporting fungal symbionts, namely, liverworts, hornworts and lycophytes, have recently been shown to form symbioses with members of ancient fungal lineages such as *Glomeromycotina* and *Mucoromycotina* (Field, Bidartondo, Rimington, *et al*. 2019). Liverworts (for example, *Marchantia* spp.) reproduce asexually by producing multicellular, roughly discoidal gemmae in gemma cups on their plant body/ thalli (Shimamura 2016). Liverwort gemmae are less than a millimetre in size and are produced in large numbers. The gemmae that are able to host AMF, once sown, can be expected to contain a single native AMF individual at its earliest colonization stage—a stage that can be identified by the red pigmentation due to the hyphae or arbuscules in the parenchyma cells of the thalli. Isolation of the AMF would involve trapping fungi in the thallus of the liverwort. While the first trapping involves isolation of the AMF with young thalli from field roots (previously colonized with multiple native AMF), the second trapping involves isolation of AMF from the thalli of first trapping with new liverwort thalli. The isolated species can be identified by various molecular techniques such as PCR, Sanger sequencing, etc. Sowing the infected thalli (mycothalli, infected with the species of interest) adjacent to a recipient plant has been found to be sufficient for rapid inoculum colonization. Further studies considering various risk factors (change of genotype during host plant shift, recombination of co-existing nucleotypes, intraspecific nucleotype change, and so on) under different growth conditions (soil type, soil nutrients, microbes, temperature, and so on) with different source roots and recipient host plants would allow a deeper understanding of using liverwort gemmae as potential AMF propagules (Kobae, Ohtomo, Morimoto, *et al*. 2019).

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Recent references

The latest additions to the network's database on mycorrhiza are published here for the members' information. The list consists of papers from the following journals:

- *Agronomy*
- *Annual Review of Phytopathology*
- *Archives of Microbiology*
- *BMC Plant Biology*
- *Ecology and Evolution*
- *Frontiers in Microbiology*
- *Frontiers in Plant Science*
- *Geoderma*
- *Global Advanced Research Journal of Microbiology*
- *Journal of Basic Microbiology*
- *Journal of Photochemistry and Photobiology B: Biology*
- *Mycorrhiza*
- *Nature Plants*
- *New Phytologist*
- *Plant Direct*
- *Rhizosphere*
- *Science of the Total Environment*
- *Scientific Reports*
- *Tree Physiology*
- *Trends in Plant Science*
- *Water*

Name of the author(s) and year of publication	Title of the article, name of the journal, volume number, issue number, page numbers (address of the first author or of the corresponding author, marked with an asterisk)
Cosme M*, Fernández I, Van der Heijden MGA, Pieterse CMJ. 2019	**Non-mycorrhizal plants: the exceptions that prove the rule** *Trends in Plant Science* **23** (7): 577–587 [*Plant–Microbe Interactions, Department of Biology, Science4Life, Utrecht University, PO Box 800.56, 3508 TB Utrecht, The Netherlands]
Cristina S C Calheiros*, Sofia I A Pereira, Albina R. Franco, Paula M L Castro. 2019	**Diverse arbuscular mycorrhizal fungi (AMF) communities colonize plants inhabiting a constructed wetland for wastewater treatment** *Water* **11** (8): 1535 [Universidade Católica Portuguesa, CBQF–Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal]
Faggioli V*, Menoyo E, Geml J, Kemppainen M, Pardo A, Salazar MJ, Becerra AG. 2019	**Soil lead pollution modifies the structure of arbuscular mycorrhizal fungal communities** *Mycorrhiza* **29** (4): 363–373 [*Instituto Nacional de Tecnología Agropecuaria, EEA Marcos Juárez, Ruta 12 km 36, 2580, Marcos Juárez, Argentina]
Fei Wang*, Michael A. Kertesz, Gu Feng. 2019	**Phosphorus forms affect the hyphosphere bacterial community involved in soil organic phosphorus turnover** *Mycorrhiza* **29** (4): 351–362 [*College of Resources and Environmental Sciences, China Agricultural University, Beijing, People's Republic of China]
Gavito ME*, Jakobsen I, Mikkelsen TN, Mora F. 2019	**Direct evidence for modulation of photosynthesis by an arbuscular mycorrhiza-induced carbon sink strength** *New Phytologist* **223** (2): 896–907 [*Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Universidad Nacional Autónoma de México, Antigua Carretera a Pátzcuaro No. 8701, Col. Ex Hacienda de Sán José de la Huerta, Morelia, 58190, Michoacán, México]

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Name of the author(s) and year of publication	Title of the article, name of the journal, volume number, issue number, page numbers (address of the first author or of the corresponding author, marked with an asterisk)
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Forthcoming events
conferences, congresses, seminars, symposiums, and workshops

Puducherry, **India** November 7–9, 2019	**National Conference on 'Recent Advances in Biodiversity, Biology and Biotechnology of Fungi' and 46th Annual Meeting of Mycological Society of India (MSI)** *E-mail*: sarmavv@yahoo.com; msi2019pu@gmail.com *Website*: http://fungiindia.co.in/images/2019/2019.pdf
Madrid, **Spain** November 11–12, 2019	**3rd Annual Congress on Soil, Plant, and Water Sciences** *E-mail*: soilscience@expertsgathering.com *Website*: https://soilscience.insightconferences.com/events-list/soil-and-plant-ecology
Amsterdam, **The Netherlands** November 14–15, 2019	**23rd World Congress on Biotechnology** *E-mail*: biotechnology@meetingsnexpo.com *Website*: https://biotechnologycongress.conferenceseries.com/
Haryana, **India** November 15–18, 2019	**60th Annual Conference of Association of Microbiologists of India and International Symposium on Microbial Technologies in Sustainable Development of Energy, Environment, Agriculture, and Health** *E-mail*: ami2019cuh@gmail.com *Website*: http://www.ami2019cuh.com/
Rome, **Italy** November 18–19, 2019	**7th Global Conference on Applied Microbiology and Biotechnology** *E-mail*: appliedmicrobiology2019@outlook.com *Website*: http://kindcongress.com/congress/microbiology-and-biotechnology-2019/
Edinburg, **Scotland** November 18–19, 2019	**4th International Conference on Applied Microbiology and Beneficial Microbes** *E-mail*: appliedmicrobes@asiapacificmeets.com; microbiology@expert-meetings.com *Website*: https://appliedmicrobiology.conferenceseries.com/
Singapore City, **Singapore** November 25–26, 2019	**3rd International Conference on Plant and Soil Science** **Theme: The New Distinct Horizontal Layers Help To Take Innovations In Plant With Changing Environment** *E-mail*: soilscience@insightsummits.com *Website*: https://www.meetingsint.com/conferences/plant-soilscience
La Trobe University, **Melbourne** November 26–29, 2019	**Australian Society of Plant Scientists Conference (ASPS 2019)** *E-mail*: k.johnson@latrobe.edu.au *Web site*: https://www.asps.org.au/combio/asps-2019
Vancouver, **Canada** December 6–7, 2019	**2nd International Conference on Microbiome, Probiotics, and Biostimulants** *E-mail*: microbiome@eventsupporting.org *Website*: https://microbiome.conferenceseries.com/
Osaka, **Japan** February 17–18, 2020	**5th International Conference on Plant Science and Physiology** *Email*: Plantphysiology@asiameetings.org *Website*: https://plantphysiology.conferenceseries.com/
Bangkok, **Thailand** April 14–16, 2020	**7th International Conference on Food and Agricultural Sciences (ICFAS 2020)** *Email:* icfas@iacsitp.com *Website*: http://www.icfas.org/

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