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Effect of Combined Inoculation of an AM Fungus with Soil Yeasts on Growth and Nutrition of Cowpea in Sterilized Soil

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Abstract: A glasshouse experiment was conducted to study the effect of combined inoculation of AM fungus, *Glomus mosseae* and soil yeasts on growth and nutrition of cowpea. Single inoculation of mycorrhizal fungus *Glomus mosseae* increased growth, nutrient uptake and growth of cowpea. The growth enhancement was much more pronounced in presence of soil yeasts tested *viz. Rhodotorula mucilaginosa*, *Metschnikowia pulcherrima*, *Trichosporon cutaneum* var. *cutaneum*, *Saccharomyces cerevisiae*, *Cryptococcus laurentii*, *Debaryomyces occidentalis* var. *occidentalis* and highest effect was seen on inoculation with *Glomus mosseae* and *Saccharomyces cerevisiae*. The same combination resulted in highest phenolic and chlorophyll content of the plant, increased mycorrhizal root colonisation, AM spore numbers and yeast population in the root zone soil.

Key words: Cowpea · *Glomus mosseae* · mycorrhiza · *Saccharomyces cervisiae* · soil yeasts

INTRODUCTION

Mycorrhizal fungi are recipients of wide attention as part of popular paradigm that considers an active and diverse soil biological community, essential for increased sustainability of agricultural systems [1]. Mycorrhizal fungi are known to influence plant growth by increased uptake of diffusion limited nutrients and water mineral uptake [2, 3], increased phenolic content to inhibit the growth of pathogens [4, 5] and increased chlorophyll content thus helping in increased photosynthesis [6, 7].

The development of AM fungi in plant roots and their effects on plant growth are influenced by the indigenous AM fungi present in the soil which may compete with the introduced AM fungi [8]. Mycorrhizal fungi interact with a wide range of other soil organisms in the root, rhizosphere and in the bulk soil. These interactions may be inhibitory or stimulatory; some are clearly competitive and others may be mutualistic. The synergestiic interaction of AM fungi with *Rhizobium*, *Azospirillum* [9, 10] and *Azotobacter* [11] in several economically important crop plants have been well established. Though, AM fungi are not capable of fixing atmospheric N_2, they are known to increase N_2-fixation and positively interact with N_2- fixers [12, 13]. Combined inoculation of phosphate solubilizing microorganisms and AM fungi have shown better N and P uptake and improved crop yields in nutrient deficient soils [14]. A synergestic relationship between saprophytic fungus *Fusarium oxysporum* and AM fungi resulting in increased root colonization and enhanced plant growth has been observed [15].

Enhancement of root colonization of legumes by native AM fungi through inoculation of seeds or soil with yeasts *Saccharomyces cerevisiae* and *Rhodotorula mucilaginosa* has been reported by earlier workers [14, 16]. Yeasts are one of the important sources of Vitamin B_{12} which is exclusively of microbial origin. Plants do not require Vitamin B_{12} for their growth. Though Vitamin B_{12} produced by yeasts may not be required directly for plant growth, yeasts in the root zone may influence plant growth indirectly by encouraging the growth of other plant growth promoting rhizomicroorganisms (PGPRs). Since the information on interaction between AM fungi and yeasts is sparse, the present study was undertaken to understand the interaction between yeasts and AM fungi in sterilized soil using cowpea as the test plant, under glass house conditions.

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MATERIALS AND METHODS

The soil used in the experiment was an Alfisol of a fine kaolinitic, isohyperthermic Typic Kanhaplustalfs type with a pH of 5.8, available P of 5.6 mg g^{-1} (NH4F + HCl extractable) and indigenous mycorrhizal spore count of 37 per 50 g of soil. Cowpea seeds (Variety TC-201) were surface sterilized with 70 percent ethyl alcohol for 1 minute which was followed by washing in sterile distilled water. Seeds were then treated with 0.01 % $HgCl_2$ for three minutes and washed with sterile distilled water 6 times. Cowpea seeds were sown in pots containing soil and were thinned to 2 plants per pot after one week of germination. The plants were watered once in two days and were grown for 90 days under glasshouse conditions.

Glomus mosseae, a local isolate obtained from the rhizosphere of *Leucaena leucocephala,* maintained as pot culture in sterilized sand: soil (1:1 v/v) mixture on Rhodes grass (*Chloris gayana*) was used as mycorrhizal culture in the present study. The air-dried inoculum contained AM hyphae, spores and root pieces. About 12,500 infective propagules based on the most probable number estimation [17] was added as thin layer, 3 cm below the soil surface of each seeding hole.

Six yeast cultures namely *Rhodotorula mucilaginosa* (MTCC 850), *Metschnikowia pulcherrima* (MTCC 632), *Trichosporon cutaneum var. cutaneum* (MTCC 255), *Saccharomyces cerevisiae* (MTCC 170), *Cryptococcus laurentii* (MTCC 3953), *Debaryomyces occidentalis var. occidentalis* (MTCC 29) were procured from the Microbial Type Culture collection, Institute of Microbial Technology, Chandigarh, India. The yeast cultures were grown in malt yeast extract broth for 6 days at 30°C in a shaker at 120 rpm. The cultures were centrifuged at 5,500 rpm for 20 min and the supernatant discarded and the pellets containing yeast cells were suspended in 0.01 M $MgSO_4$ solution. The colony-forming units (CFU) of soil yeasts were 8-10 x 10^5/ml inoculum. The yeast cultures were applied as per the treatment to the seed hole at the rate of 25 ml per hole. The control plants were provided with 25 ml of 10 mM $MgSO_4$ solution.

Cow pea seeds (Variety TC-201) were obtained from Indian Council of Agricultural Research coordinated project on arid legumes, University of Agricultural Sciences, Bangalore and seeds were surface sterilized with 70 per cent ethyl alcohol for 1 minute followed by washing three times with sterilized distilled water. Seeds were later treated with 0.01% $HgCl_2$ for three minutes and washed with sterilized distilled water six times. Cowpea seeds were sown in pots of 2 kg capacity containing sand:soil mix (1:1 v/v). The soil used in the study was a fine kaolinitic, isohyperthermic, kanhaplustaffs type with pH of 5.7 and available P of 5.6 ppm, (NH_4F+HCl extractable) with an indigenous AM spore population of 38 per 50 g soil. The substrate was sterilized by autoclaving twice at 20 lbs pressure for 1 hour. Five seeds were sown in each pot and were thinned to two plants per pot after one week of germination. The treatments were imposed at the time of sowing of seeds. Plants were maintained under glasshouse conditions and were watered once in two days and were grown for 90 days.

The experiment consisted of the following 8 treatments with 8 replications 1. Uninoculated control and inoculated treatments with 2. *Glomus mosseae* (Gm) 3. Gm + *Rhodotorula mucilaginosa* 4. Gm + *Metschnikowia pulcherrima* 5. Gm + *Trichosporon cutaneum* var. *cutaneum 6.* Gm + *Saccharomyces cerevisiae* 7. Gm + *Cryptococcus laurentii* 8. Gm + *Debaryomyces occidentalis* var. *occidentalis.*

Plant height and number of leaves were recorded once in 20 days interval up to the time of harvest, but only the values at the time of harvest are presented in this paper. Plants were harvested 90 days after sowing. Pod yield per plant was recorded. Dry weights of shoot and root (after washing the soil) separately were determined after drying to a constant weight at 60°C. The chlorophyll content of leaves was estimated by Dimethyl sulfoxide (DMSO) method [18]. The phenolic content of root and leaf samples was estimated by the method given by Sadasivam and Manickam [19]. Plant phosphorus concentration was estimated by the vanadomolybdate yellow colour method [20]. The nitrogen content of the plant samples were determined by the method given by Catalado *et al.* [21].

Mycorrhizal root colonization was determined by the grid line intersect method [22] after staining the roots with trypan blue [23]. The number of extramarital chlamydospores produced by the AM fungus in the root zone soil was estimated by wet sieving and decantion method [24]. The population of soil yeasts in the root zone soil was determined at 20 days interval by serial dilution plate method using malt extract agar [25] but the values at the time of harvest only presented in this paper. The vitamin B_{12} production of yeasts was done by the microbiological assay using *Lactobacillus delbrueckii* sub sp. *lactis* (MTCC 0911) as the test organism [26].

The results of various parameters obtained from the experiments were analysed by statistical analysis suitable to Randomised Complete Block Design and were separated using Duncan's Multiple Range Test (DMRT) [27].

RESULTS AND DISCUSSION

The height of the plants inoculated with *G. mosseae* was significantly different from uninoculated control (Table 1). Highest plant height was observed in the treatment *G. mosseae* + *S. cerevisiae* which was statistically on par with *G. mosseae* + *Metschnikowia pulcherrima* but different from *G. mosseae* alone and uninoculated control. Plants inoculated with *G. mosseae* + *Saccharomyces cerevisiae* had maximum number of leaves which was on par with *G. mosseae* plus all the other soil yeasts combinations but significantly different from *G. mosseae* alone and control, the two being statistically on par with each other (Table 1).

Single inoculation with *G. mosseae* resulted in a shoot dry weight significantly different from that of uninoculated plants (Table 1). Shoot dry weight was maximum in plants treated with *Glomus mosseae* + *Saccharomyces cerevisiae* followed by *G. mosseae* + *Rhodotorula mucilaginosa* which did not differ significantly from each other. The other treatments with *G. mosseae* + soil yeasts, yielded better and significantly different shoot dry weight compared to single inoculation of *G. mosseae*. Root dry weight was also maximum in plants inoculated with *G. mosseae* + *S. cerevisiae* which was statistically on par with the treatments *G. mosseae* + *R. mucilaginosa*, *G. mosseae* + *Trichosporon cutaneum* and *G. mosseae* + *Metschnikowia pulcherrima*. Inoculation with *G. mosseae* alone resulted in significantly higher root dry weight compared to uninoculated control.

The pod yield per plant also showed almost a similar trend, highest yield being in plants treated with *G. mosseae* + *S. cerevisiae* followed by *G. mosseae* + *R. mucilaginosa* and *G. mosseae* + *T. cutaneum* which were on par with each other. Yield of plants inoculated with *Glomus mosseae* alone and uninoculated control plants did not differ significantly (Table 1).

It is now well established that arbuscular mycorrhizal fungi improve growth and nutrition of several plants that are important in agriculture, horticulture and forestry [28]. Several workers have reported growth enhancement of crop plants because of *G. mosseae* inoculation [29, 30]. Similar results were obtained in the present study also. The synergestic interaction between AM fungi and beneficial soil microorganisms like *Rhizobium, Azospirillum* and *Azotobacter* and their consequential beneficial effects on economically important crop plants is also well documented [9, 10, 30, 31]. An increase in plant biomass due to inoculation with the native AM fungi + *S. cerevisiae* has been reported by Singh *et al.* [14]. This was attributed to the stimulatory effect of yeasts on multiplication, spore germination and establishment of native AMF.

Nutrient uptake: Plants inoculated with *G. mosseae* alone had significantly higher shoot P content than the uninoculated plants. Shoot P uptake was highest in plants inoculated with *G. mosseae* + *S. cerevisiae* which was on par with *G. mosseae* + *R. mucilaginosa* followed by *G. mosseae* + *D. occidentalis*. (Table 2). A similar trend was observed for root P, maximum in treatment with *G. mosseae* + *S. cerevisiae* and least in uninoculated control. AM fungi are known to improve P nutrition of plants especially in P deficient soil and can translocate phosphate by scavenging a larger volume of soil with extensive hyphae [3, 32]. The present study brings out that coinoculation with yeasts further enhances P uptake by AM fungi.

Highest shoot N was observed in plants inoculated with *G. mosseae* + *S. cerevisiae* which differed significantly from all other treatments. Regarding N content of the shoot, plants inoculated with *G. mosseae* alone had significantly higher content compared to uninoculated plants. Root N content also showed a similar trend (Table 2).

Table 1: Response of cowpea to inoculation with *G. mosseae* and yeasts in sterilized soil

Treatments	Height	No of leaves	Shoot dry weight	Root dry weight	Pod Yield(g/Plant)
Uninoculated control	21.69^{e}	7.0^{c}	3.73^{f}	1.32^{d}	9.11^{d}
Inoculated with *G. mosseae* (Gm)	23.46^{d}	7.4^{bc}	4.2^{e}	1.58^{c}	10.03^{d}
Inoculated with Gm + *R. mucilaginosa*	29.79^{b}	9.2^{ab}	6.05^{a}	2.00^{a}	13.1^{ab}
Inoculated with Gm + *M. pulcherrima*	30.84^{ab}	8.7^{abc}	5.81^{b}	1.9^{ab}	10.1^{d}
Inoculated with Gm + *T cutaneum* var. *cutaneum*	29.09^{b}	9.0^{ab}	5.30^{c}	1.95^{ab}	12.4^{abc}
Inoculated with Gm + *S. cerevisiae*	31.84^{a}	10.1^{a}	6.13^{a}	2.08^{a}	13.2^{a}
Inoculated with Gm + *C. laurentii*	25.86^{c}	9.2^{ab}	4.71^{d}	1.70^{bc}	11.83^{bc}
Inoculated with Gm + *D. occidentalis* var. *occidentalis*	23.76^{d}	9.4^{a}	5.71^{b}	1.68^{bc}	11.7^{c}

Means having same superscript do not differ significantly at P=0.05level by DMRT

Table 2: Effect of *Glomus mosseae* and yeasts on nitrogen and phosphorus uptake of cowpea

Treatments	Shoot P content (mg/plant)	Root P Content (mg/plant)	Shoot N (mg/plant)	Root N (mg/plant)
Uninoculated control	0.58^{f}	0.17^{d}	13.58^{h}	3.99^{g}
Inoculated with *G. mosseae* (Gm)	0.69^{e}	0.29^{c}	15.96^{g}	4.80^{f}
Inoculated with Gm + *R. mucilaginosa*	1.08^{ab}	0.45^{a}	23.59^{b}	6.30^{b}
Inoculated with Gm + *M. pulcherrima*	0.98^{c}	0.43^{ab}	22.66^{c}	5.87^{d}
Inoculated with Gm + *T cutaneum* var. *cutaneum*	0.96^{c}	0.45^{a}	20.67^{e}	6.14^{c}
Inoculated with Gm + *S. cerevisiae*	1.15^{a}	0.50^{a}	24.52^{a}	6.64^{a}
Inoculated with Gm + *C. laurentii*	0.85^{d}	0.37^{bc}	18.37^{f}	5.20^{e}
Inoculated with Gm + *D. occidentalis* var. *occidentalis*	1.01^{bc}	0.36^{bc}	21.69^{d}	5.17^{e}

Means having same superscript do not differ significantly at P=0.05level by DMRT

Table 3: Effect of *Glomus mosseae* and yeast inoculation on chloroophyll and phenolic content of cow pea

Treatments	Chlorophyll content (mg/g)	Phenolic content (mg g^{-1}) leaves	Phenolic content (mg g^{-1}) root
Uninoculated contro	l0.410^{e}	10.88^{e}	0.87^{d}
Inoculated with *G. mosseae* (Gm)	0.418^{e}	11.13^{d}	1.00^{cd}
Inoculated with Gm + *R. mucilaginosa*	0.480^{ab}	11.38^{c}	1.62^{b}
Inoculated with Gm + *M. pulcherrima*	0.428^{bc}	11.38^{c}	1.00^{cd}
Inoculated with Gm + *T cutaneum* var. *cutaneum*	0.440^{abc}	11.13^{d}	1.12^{c}
Inoculated with Gm + *S. cerevisiae*	0.491^{a}	15.00^{a}	2.30^{a}
Inoculated with Gm + *C. laurentii*	0.460^{abc}	11.25^{cd}	1.00^{cd}
Inoculated with Gm + *D. occidentalis* var. *occidentalis*	0.483^{a}	11.75^{b}	1.50^{b}

Means having same superscript do not differ significantly at P=0.05level by DMRT

Table 4: Effect of yeast inoculation on microbial parameters of cow pea grown in sterilized soil

Treatments	Root colonisation (%)	Spore count/ 50g soil	Soil yeasts population (CFUX10^{4})
Uninoculated control	0.0^{f}	0.0^{f}	0.0^{g}
Inoculated with *G. mosseae* (Gm)	66.0^{e}	28.0^{e}	0.0^{g}
Inoculated with Gm + *R. mucilaginosa*	72.7^{c}	32.0^{de}	80.2^{b}
Inoculated with Gm + *M. pulcherrima*	70.7^{cd}	51.3^{a}	40.5^{d}
Inoculated with Gm + *T cutaneum* var. *cutaneum*	69.7^{d}	44.0^{bc}	70.5^{c}
Inoculated with Inoculated with Gm + *S. cerevisiae*	81.0^{a}	53.0^{a}	90.5^{a}
Inoculated with Gm + *C. laurentii*	78.0^{b}	38.0^{cd}	21.2^{e}
Inoculated with Gm + *D. occidentalis* var. *occidentalis*	69.0^{d}	47.7^{ab}	14.0^{f}

Means having same superscript do not differ significantly at P=0.05level by DMRT

Shoot and root N content of plants inoculated with *G. mosseae* + *S. cervisiae* was 1.7 times more compared to uninoculated plants. Singh *et al.* [14] reported increased nodule number and dry weight of legumes due to inoculation with yeasts because of stimulation of indigenous rhizobia. Since the present investigation is in sterilized soil, the increase in N content with yeast inoculation may not be due to its effect on the proliferation of native rhizobia but could be due to stimulatory effect of yeast on spore germination and multiplication of AM fungi [14] which are known to increase the uptake of P and other micronutrients involved in N fixation [12, 13].

Chlorophyll and phenolic content: Increase in the chlorophyll content due to inoculation with AM fungi in plants has been reported by earlier workers [6, 7]. Such an increase because of *G. mosseae* alone was not

observed in the present study. The treatment *G. mosseae* + *S. cerevisiae* resulted in significantly higher leaf chlorophyll content which was on par with *G. mosseae* + all other soil yeast combinations except *G. mosseae* + *M. pulcherrima* (Table 3).

Thus the present study brings out that co-inoculation of soil yeasts with the AM fungus can significantly enhance the chlorophyll content of leaves compared to inoculation with the AM fungus alone. Plants treated with *G. mosseae* + *S. cerevisiae* had the highest phenolic content in leaves which differed significantly from all other treatments. Single inoculation with *G. mosseae* also resulted in significantly different phenolic content compared to the uninoculated control (Table 3). Phenolic contents of the root also followed more or less a similar trend except that the content in *G. mosseae* alone and uninoculated treatments did not differ significantly. Increased polyphenol oxidase, phenylalanine ammonia lyase and peroxidase activity in mycorrhizal plants, which are responsible for the oxidation of phenolic compounds to quinones to inhibit pathogens growth is well documented [4, 5].

Mycorrhizal parameters and yeast population: *G. mosseae* alone treated plants had significantly higher root colonization compared to uninoculated plants which showed zero colonisation (Table 4). Coinoculation of yeast along with *G. mosseae* significantly enhanced mycorrhizal root colonisation than inoculation with *G. mosseae* alone. Plants inoculated with *G. mosseae* plus *S. cerevisiae* showed maximum mycorrhizal root colonization. Mycorrhizal spore numbers in the root zone soil almost followed a similar trend. Highest mycorrhizal spore numbers were encountered in the root zone soil of plants inoculated with *G. mosseae* + *S. cerevisiae* which was on par with the treatment *G. mosseae* + *M. pulcherrima*. Singh *et al.* [14] observed that inoculation of legumes with the yeast *S..cerevisiae* increased production of vesicles, arbuscules and spores by the indigenous mycorrhizal fungi while Fracchia *et al.* [15] reported enhanced AM colonization of soyabean and red clover when *R. mucilaginosa* was applied to the soil.

The root zone soil samples from the uninoculated and *G. mosseae* alone treated plants did not show any yeast population, while yeast population occurred in all dual inoculation treatments. Highest yeast population occurred in the treatment *G. mosseae* plus *S. cerevisiae* followed by the treatment *G. mossease* plus *R. mucilaginosa* (Table 4). This confirms the observations made by earlier workers that AM fungi stimulated the activity of beneficial soil microorganisms like *Azotobacter* [33] and plant growth-promoting rhizobacteria [30, 31] in the rhizosphere.

Vitamin B_{12} production by soil yeasts: An exhibition zone of approximately 5 mm was produced by all yeast cultures in *Lactobacillus delbrueckii* seeded plates suggesting that all of them produce vitamin B_{12}. Neither yeasts nor Vitamin B_{12} produced by them seems to have any direct effect on plant growth and yield [34]. At the same time, Singh (personal communication) observed that inoculation with *Saccharomyces cerevisiae* had negligible effect on non- mycorrhizal plants while it increased the root colonization and spore count of mycorrhizal plants. This suggests that the yeasts stimulate colonisation of roots by AM fungi there by indirectly helping the nutrition and growth of plants, upholding the views expressed by Larsen and Jacobsen [35].

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