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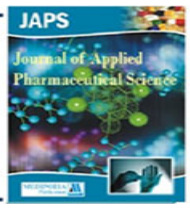

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Comparative effect of *Trichoderma hamatum* and host-specific *Rhizobium species* on growth of *Vigna mungo*

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ABSTRACT

The present study was designed to investigate the combine effect of *Trichoderma hamatum* and host-specific *Rhizobium sp.* of *Vigna mungo* on growth and biochemical parameters of same legume. The results proved that *T.hamatum* and host-specific *Rhizobium sp* are compatible with each other and their combine use was found effective not only in improving the growth parameters including lengths of roots & shoots and fresh biomass of experimental crop but also increasing the total chlorophyll, carbohydrate and crude protein contents as compared to control plants ($p<0.05$). Similarly, the amount of both nitrogen and phosphorus was significantly increased in leaves of the same legume ($p<0.05$). Therefore, *T.hamatum* could be a good alternate of chemical fertilizer and fungicide for improving the growth and productivity of *V.mungo*.

Keywords: *Trichoderma hamatum, Rhizobium species, Vigna mungo.*

INTRODUCTION

Trichoderma species including *T.viride, T.harzianum, T.hamatum, T. koningi, T.pseudokoningi,* etc, are common residents of rhizosphere (soil adhering to root surface) and rhizoplane (root surface) of plants (Mishra, 1996). These are reported as endophytic saprophytes as they readily colonies the root surface or cortex of host plant (Harman et al., 2004). This *Trichoderma*-plant association, instead of producing deleterious effect, it benefits the host plant in health, growth and productivity (Harman, 2006). Species of this genus are well-reported as biocontrol agents against several fungal pathogens through mechanisms such as mycoparasitism (mycelial coiling), antibiosis, cell wall degrading enzymes and induced resistance in host plant against diseases by altering plant gene expression (Pandya and Saraf, 2010; Alfano et al., 2007). Studies showed that *T. hamatum* 382 can reduce the occurrence of foliar diseases of several vegetable crops including tomato by altering genes involved in stress and protein metabolism (Al-Dahmani *et al.,* 2005; Khan *et al.,* 2004; Horst *et al.,* 2005).

On the other hand, *Trichoderma sp*. are also reported as growth promoting fungi by enhancing the availability of nutrients and minerals (Fe, N, P) for plants, producing plant growth hormones and decomposing organic material (Kaewchai *et al.,* 2009). In addition *Trichoderma species* helped plants to withstand against abiotic stresses such as by increasing the length of secondary roots deep in the ground or soil and improving the water holding capacity to provide protection against drought (Mastouri *et al*., 2010). In this regard, *T.hamatum* was recently reported to induce tolerance in cocoa plants against water deficit through increasing root growth (Bae *et al.,* 2009).

Interestingly, the growth promoting effect of *Trichoderma sp* is not restricted to non-legumes but these have also been reported to enhance many growth parameters with improved yield of legume crops (Hoyos-Carvajal *et al.,* 2009). There are several *Trichoderma* products commercially available in market as fungal biofungicides and biofertilizers which are successfully used not only to control plant diseases but also to promote the growth and development of plants in greenhouse and field production (Kaewchai *et al.,* 2009). The present study was designed to investigate the effect of *Trichoderma hamatum* alone and in combination with host-specific *Rhizobium sp.* of *Vigna mungo* on growth and biochemical parameters of same legume.

MATERIAL AND METHODS

Experimental Crop

Seeds of *Vigna mungo* L. (Mash) were purchased from Old vegetable market, Hyderabad, Pakistan.

Fertilizer and Fungicide

Fertilizer NPK and fungicide carbendazim were purchased from dealer of Agrochemical, Old vegetative market, Karachi, Pakistan and were used as positive controls in a concentration of 2500 ppm each.

Sources of *Trichoderma hamatum* and *Rhizobium species*

Root sample of wild herb *Amaranthus viridis* (family: *Amarantheceae*) from north nazimabad, Karachi and used to isolate *Trichoderma hamatum* from rhizoplane by using standard method (Aneja, 1993). In which roots were washed in running tap water, 1cm long root pieces were cut from tap and lateral roots and washed in sterilized distilled water. Then root pieces were transferred on plate containing potato dextrose agar (PDA) incorporated with penicillin (100,000unit/liter) and streptomycin (0.2g/liter) to inhibit the growth of gram-positive and negative bacteria. Petri plates were incubated for 5 days at 28°C. Grown fungi were identified by expert of Botany Department, University of Karachi, Karachi, Pakistan. Of which, *T. hamatum* was made separated, isolated pure and preserved on PDA slants for further use.

Where as root samples of *Vigna mungo* (family: *Fabaceae*) were collected from net house of Jinnah University for Women and used to isolate *rhizobial* culture by crushed-nodule method (Aneja, 1993). In which, roots were washed in running tap water to remove adhering soil particles. Select healthy pink, unbroken and firm nodules. Immerse the nodules in 0.1% $HgCl_2$ for 5 minutes for surface sterilization. Then nodules were washed with sterilized water thrice. Place the nodules in 70% ethyl alcohol for 3 minutes and washed them again with sterilized distilled water. Nodules were crushed in sterilized distilled water (1 mL) to make uniform suspension of *rhizobia* that referred as nodule extract. Make serial dilutions of nodule extract (1:10 to 1:10,000). Spread 0.5mL of each of last two highest dilutions on Yeast Extract Mannitol Agar (YEMA) plates and incubate at 26°C for 10 days. Large gummy colonies of *rhizobia* were appeared within 4-5 days. The *rhizobial* isolates were sub-cultured, purified and tested for their ability to form nodules on *vigna mungo* (Beck *et al.,* 1993). The host-specific *Rhizobium sp* were purified and maintained on YEMA slants and stored at 4 to 8°C for further use in the present study.

Preparation of Conidial and cell inoculums of *T.hamatum* and *Rhizobium sp*

Four petri plates containing five day old cultures of *T.hamatum* on PDA were blended with 40mL of distilled water (10mL/petri plate), then make its volume up to 50mL with the help of distilled water and considered it 1:10 dilution. Then make its serial dilutions from 1:100 to 1:10,000. Twenty five milliliters of highest dilution was used as inoculums after calculating number of conidia per mL with help of haemocytometer. Similar procedure was used to prepare cell inoculums of *Rhizobium sp.* after calculating number of cells per mL.

Experimental procedure

The randomized complete block designed pot experiment was conducted in net house of Department of Botany, Jinnah University for Women, Nazimabad Karachi, Pakistan in 2010 to check the effects of *T.hamatum* alone and in combination with host-specific *Rhizobium sp.* on the growth of *vigna mungo*. Seeds of *V. mungo* were sown in pots filled with 2 kg soil each. After 5 days of germination, developing seedlings in each pot were initially inoculated with different treatments. Twenty five milliliters of suspension of each treatment (approximately 1.2×10^6 conidia/mL of *T.hamatum* and 1.9×10^8 cells/mL of *Rhizobium sp.*) were used. Five replicates were used for each treatment. During the first few days after inoculation, care should be taken in watering the plants to avoid the washing the inoculums out of the soil and then watering was done on alternate days. Five plants of each treatment (1 plant/replicate/treatment) were uprooted at 30^{th} and 60^{th} day of growth to measure the selected physical and biochemical parameters.

Physical Parameters

Root length was measured from the point of attachment of the stem base to the tip of the adventitious root. Where as shoot length was measured from the stem base to the tip of the longest leaf stretched and plant fresh weight (biomass) was recorded through electrical balance.

Biochemical Analysis

Biochemical parameters were estimated in leaves of experimental plants. Total chlorophyll & its fractions (a, b) and total carbohydrate contents were determined by methods described by Arnon and Yemm & Willis (Arnon, 1949; Yemm and Wills, 1956) while crude protein by multiplying percent nitrogen value through 6.25 (Sriperm *et al.,* 2011). The percent nitrogen and phosphorus were estimated by Nesseler's (Singh, 1982) and Braton reagent (Ashraf *et al.,* 1992).

Statistical analysis

Results of present pot experiment are expressed as mean ± SD. Data was analyzed by *One-way* ANOVA followed by Least significant difference (LSD) test by using SPSS 16 (version 4). The differences were considered significant at $p<0.05$ when treatments' mean compared with control.

RESULTS AND DISCUSSION

Beans are considered as high protein with low fat diet and are widely used in developing countries especially Asian countries as staple food or as a substitute of animal protein (Tresina *et al.,* 2010). However legumes are subjected to many fungal pathogens that severely affect its roots and leaves and become as major limiting factor for the yield of these crops in many countries (Puglia and Aragona, 1997). In order to increase the yield of legumes, chemical fertilizers and fungicides are the first preference of farmers but their use become limited because of economical, environmental and health reasons (Shaban and El-Bramawy, 2011). Now-a-days microbial inoculants have been using as an alternative of commercially available chemicals to control diseases and promote growth of both legume and non-legume plants (Nakkeeran *et al.,* 2002).

The biocontrol and growth promoting potentials of *Trichoderma species* have been extensively studied (Pandya and Saraf, 2010). Similarly *Rhizobium species* are well-documented for their abilities to fix atmospheric nitrogen in roots of legume plants through nodulation which induced significant growth promoting and yielding effects in their specific host plants (Mia and Shamsuddin, 2010). These are also reported to restrict the growth of many soil-borne pathogens of legume plants such as *Rhizoctonia solani, Fussarium oxysporum, F.solani, Macrophomina phaseolina*, etc, which also in turn produce health improving effects on plants (Sessitsch *et al.,* 2002). In the present study, according to the observed growth parameters of *V. mungo* (Table 1), *T. hamatum* alone increased the shoot and root lengths at 30th and 60th day of germination respectively while did not produce any effect on fresh weight of crop on same time intervals as compared to host-specific *rhizobium* sp. of *V.mungo* which produced significant effect on all growth parameters including root & shoot lengths and fresh weight of plants. However, *T.hamatum* in combination with host-specific *rhizobium sp*. significantly increased growth performance of *V.mungo* at both days of harvesting as compared to groups of plants treated with fertilizer, fungicide individually, *Rhizobium sp* with NPK (fertilizer) and carbendazim (fungicide). Similarly, *T.hamatum* along with fertilizer was also found effective in improving the growth performance of *V.mungo* at 60th day of its germination.

Out of all treatments, *Rhizobium sp* alone and in combination with fertilizer and fungicide significantly increased total chlorophyll content and its fractions in leaves of experimental crop. Almost similar significant effect was also observed in plants treated with *T.hamatum* with fertilizer but at 60th day of their germination. *T.hamatum* alone and in combination with *rhizobium sp* significantly increased chlorophyll content in crop at 30th day of germination (Table 2). On the basis of obtained results regarding with total carbohydrate and crude protein contents, again *T.hamatum* in combination with *rhizobium sp* was found significantly effective in increasing the amount of both these parameters at 30th and 60th day of germination. Where as *T.hamatum* and *Rhizobium sp* individually in their respective group increased carbohydrate and crude protein contents at 60th day of germination of *V.mungo*. Of course host-specific *rhizobium sp* in combination with fertilizer and fungicide was found capable in this respect (Table 3). Similarly, *T.hamatum* alone was found increasing the percent nitrogen (N %) in experimental crop at 30th day but it significantly increased percent nitrogen and phosphorus (P %) both when used with *rhizobium* sp at 30th and 60th day. *Rhizobium sp* alone and in combination with fertilizer and fungicide found effective in improving these trace elements in crop (Table 4). According the obtained results, it has been observed that *T.hamatum* and host-specific *Rhizobium sp* of *V.mungo* are compatible with each other and their combination was found effective not only in improving the growth of experimental crop as compared to control plants which did not treat with any of these but also increase the total chlorophyll, carbohydrate and crude protein contents. Similarly, trace element including nitrogen and phosphorus was significantly increased in test plants treated with the combination of *T.hamatum* and *V.mungo*-specific *Rhizobium sp*. Our findings are also comparable to the study that described the improvement in growth, nutrient uptake and yield of chickpea under glasshouse and field experiments due to the combine inoculation of *Rhizobium sp* with *Trichoderma spp* (Rudresh *et al.,* 2005). Similarly, another study showed that dual inoculation of *Rhizobium sp* with *T.harzianum* not only provide biological control against damping off and root-rot diseases of legume crops including *Vicia faba, Cicer arietinum* and *Lupines terms* but also the combination was found effective in increasing growth parameters of same crop plants in greenhouse experiments (Shaban and El-Bramawy, 2011). Therefore, *T.hamatum* can be used as a good substitute of chemical fertilizer and fungicide for producing positive effect on growth of *V.mungo* by improving the soil fertility and providing disease free environment by residing in roots tissues and showing its rhizosphere competence (Hoyos-Carvajal *et al.,* 2009), this condition would more strengthen by host-specific *Rhizobium sp* of same legume that could enhanced its natural ability of competition and creating symbiosis with roots which in turn improve the growth of legume and improving the mineral (N, P) content of plants.

Table. 1: Effect of treatments on growth performance of *V. mungo*.

	Growth performance						
	30 days			60 days			
S. No.	Treatment	Root length*	Shoot length*	Fresh weight**	Root length*	Shoot length*	Fresh weight**
1	Control	27.5 ± 2.29	13.33 ± 0.76	0.674 ± 0.09	30.4 ± 0.17	18.83 ± 1.04	1.18 ± 0.39
2	JUF1	30.3 ± 1.35	20.33 ± 4.72^{c}	1.21 ± 0.50	37.4 ± 1.01^{a}	22. ±2.00	1.37 ± 0.43
3	JUR4	36.73 ± 1.36^{a}	20.06 ± 2.53^{a}	1.68 ± 0.42^{b}	38.1 ± 2.02^{a}	27.03±3.28^{a}	3.49 ± 0.19^{a}
4	FTZ	30.06 ± 1.50	20.90 ± 2.28^{b}	2.14 ± 0.13^{a}	34.9 ± 1.65^{c}	25.33 ± 0.57	2.19 ± 0.42
5	FGD	31.86 ± 1.77^{d}	21.7 ± 0.64^{a}	1.66 ± 0.16^{c}	32.33 ± 2.51	23.66 ± 3.78^{d}	1.69 ± 0.08
6	JUR4 + JUF1	36.46 ± 2.60^{a}	22.33 ± 2.46^{a}	1.73 ± 0.35^{b}	40.16 ± 4.25^{a}	24.7 ± 1.05^{c}	3.09 ± 0.41^{c}
7	JUF1 + FTZ	18.4 ± 5.7	15.96 ± 1.45	1.07 ± 0.08	37.5 ± 1.70^{a}	24.33 ± 3.21^{c}	2.61 ± 0.32^{d}
8	JUR4 + FTZ	36.63 ± 1.48^{a}	25.9 ± 1.65^{a}	1.64 ± 0.03	33.3 ± 1.08^{a}	27.06 ± 3.27^{a}	2.98 ± 0.56^{c}
9	JUR4+FGD	35.4 ± 6.02	21.5 ± 1.32^{b}	1.41 ± 0.24	36.03 ± 0.45^{a}	24.83 ± 2.01^{c}	2.1 ± 0.10

JUF1 = *Trichoderma hamatum*, JUR4 = host-specific *Rhizobium sp* of *V.mungo*, FTZ = fertilizer, FGD = fungicide. Each value is the mean ± SD (standard deviation) of 5 replicates. Any two means not sharing a superscript in common are significantly different at $p<0.05$ (LSD), * = cm, ** = gm.

Table. 2: Effect of treatments on photosynthetic pigment of *V. mungo*.

	Photosynthetic Elements					
	30 days			60 days		
S. No.	Treatment	Chl-a (mg)	Chl-b (mg)	Total Chl (mg)	Chl-a (mg)	Chl-b (mg)	Total Chl (mg)
1.	Control	0.36 ± 0.02	0.65 ± 0.05	0.81 ± 0.01	0.44 ± 0.04	0.8 ± 0.07	0.95 ± 0.07
2.	JUF1	1.36 ± 0.23	2.46 ± 0.41	2.76 ± 0.45	0.58 ± 0.11	1.05 ± 0.21	1.2 ± 0.23
3.	JUR4	0.52 ± 0.14	1.0 ± 0.36	1.17 ± 0.40^{d}	0.86 ± 0.35d	1.33 ± 0.13^{d}	2.2 ± 0.44^{a}
4.	FTZ	1.55 ± 0.08	1.7 ± 0.15	2.84 ± 0.29	0.54 ± 0.09	1.43 ± 0.46	1.55 ± 0.52
5.	FGD	1.55 ± 0.11	1.45 ± 0.13	1.68 ± 0.13	0.74 ± 0.08	1.34 ± 0.15	1.27 ± 0.55
6.	JUR4 + JUF1	1.39 ± 0.22	2.35 ± 0.50	2.84 ± 0.30	0.85 ± 0.47	1.46 ± 0.14	1.7 ± 0.52
7.	JUF1 + FTZ	0.2 ± 0.05	0.37 ± 0.09	0.42 ± 0.10	1.13 ± 0.31^{a}	1.77 ± 0.13^{a}	1.71 ± 0.58^{c}
8.	JUR4 + FTZ	0.99 ± 0.13^{a}	1.7 ± 0.11^{a}	2.02 ± 0.25^{a}	1.4 ± 0.04a	0.49 ± 0.14	2.06 ± 0.26^{a}
9.	JUR4+ FGD	0.83 ± 0.06^{a}	1.52 ± 0.14^{a}	1.69 ± 0.14^{a}	1.01 ± 0.24^{b}	1.54 ± 0.01^{c}	1.95 ± 0.44^{b}

Chl = chlorophyll, JUF1 = *Trichoderma hamatum*, JUR4 = host-specific *Rhizobium sp* of *V.mungo*, FTZ = fertilizer, FGD = fungicide. Each value is the mean ± SD (standard deviation) of 5 replicates. Any two means not sharing a superscript in common are significantly different at $p<0.05$ (LSD)

Table. 3: Effect of treatments on carbohydrates and crufde protein content of *V. mungo*.

	Biochemical Parameters			
	0 days		60 days	
S.No.	Treatment	Carbohydrates (%)	Proteins (%)	Carbohydrates (%)	Proteins (%)
1.	Control	175.3 ±11.91	9.65 ± 0.70	192.91± 14.54	10.54 ± 0.79
2.	JUF1	229.53 ±19.92	12.54 ± 1.08	328.29 ±105.28^{c}	17.94 ± 5.75^{b}
3.	JUR4	241.47 ± 63.34	13.19 ± 3.45	347.68 ±18.2b	19.0 ± 0.97^{a}
4.	FTZ	195.29 ±50.21	10.67 ± 2.74	215.90± 9.93	11.79 ± 0.53
5.	FGD	170.56 ± 5.85	9.32 ± 0.31	207.6 ± 50.49	11.34 ± 2.75
6.	JUR4 + JUF1	310.43± 39.54^{c}	16.96 ± 2.16^{b}	336.74 ±17.37^{c}	18.4 ± 0.95^{b}
7.	JUF1 + FTZ	200.63 ± 52.74	10.96 ± 2.88	219.8 ± 32.25	12.01 ± 1.76
8.	JUR4 + FTZ	514.23 ± 73.00^{a}	28.1 ± 3.98^{a}	367.8 ± 81.53^{a}	20.1 ± 4.45^{a}
9.	JUR4+ FGD	343.61 ± 49.97^{a}	18.77 ± 2.73^{a}	350.1± 20.38^{b}	19.13 ± 1.11^{a}

JUF1 = *Trichoderma hamatum*, JUR4 = host-specific *Rhizobium sp* of *V.mungo*, FTZ = fertilizer, FGD = fungicide. Each value is the mean ± SD (standard deviation) of 5 replicates. Any two means not sharing a superscript in common are significantly different at $p<0.05$ (LSD).

Table. 4: Effect of treatments on percent nitrogen and phosphorus of *V. mungo*.

	Trace Elements			
	30 days		60 days	
S. No.	Treatment	N (%)	P (%)	N (%)	P (%)
1.	Control	1.54 ± 0.1	0.11 ± 0.02	1.68 ± 0.12	0.14 ± 0.09
2.	JUF1	2.0 ± 0.17	0.18 ± 0.05	2.87 ± 0.91^{b}	0.19 ± 0.05
3.	JUR4	2.11 ± 0.79	0.16 ± 0.08	3.04 ± 0.16^{a}	0.3 ± 0.15^{d}
4.	FTZ	1.7 ± 0.44	0.44 ± 0.61^{d}	1.88 ± 0.08	0.46 ± 0.07^{a}
5.	FGD	1.49 ± 0.05	0.14 ± 0.09	1.81 ± 0.44	0.15 ± 0.01
6.	JUR4 + JUF1	2.71 ± 0.34^{b}	0.25 ± 0.17	2.94 ± 0.15^{b}	0.32 ± 0.12^{c}
7.	JUF1 + FTZ	1.75 ± 0.45	0.16 ± 0.08	1.92 ± 0.28	0.18 ± 0.05
8.	JUR4 + FTZ	4.49 ± 0.63^{a}	0.38 ± 0.07^{d}	4.68 ± 0.23^{a}	0.41 ± 0.05^{a}
9.	JUR4+ FGD	3.0 ± 0.43^{a}	0.25 ± 0.00	3.06 ± 0.18^{a}	0.37 ± 0.10^{a}

JUF1 = *Trichoderma hamatum*, JUR4 = host-specific *Rhizobium sp* of *V.mungo*, FTZ = fertilizer, FGD = fungicide. Each value is the mean ± SD (standard deviation) of 5 replicates. Any two means not sharing a superscript in common are significantly different at $p<0.05$ (LSD).

CONCLUSION

On the basis of obtained results, the combination of *T.hamatum* and host-specific *Rhizobium sp* of *V.mungo* found significantly effective in improving the growth and biochemical constituents including the total chlorophyll, carbohydrate and crude protein contents of *V.mungo*. In addition, the combination was also effective in increasing the amount of percent nitrogen and phosphorus in leaves of same crop.

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