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WHEAT GROWTH AND YIELD IN RESPONSE TO COINOCULATION OF *RHIZOBIUM, AZOSPIRILLUM AND PSEUDOMONAS* UNDER RAINFED CONDITIONS

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ABSTRACT

A wheat (GA 2002) field inoculation trial was carried out to evaluate an indigenous formulation comprising of *Rhizobium*(PAC-19/1) *Azospirillum* (ZS1) and P-solubilizing *Pseudomonas* (Psm-na) in single, dual and tripartite combination as supplement to chemical fertilizers. The effect on growth and yield indices was compared with full recommended dose of Nitrogen (N) and Phosphorus (P) at NARC experimental field area during 2006-07. The bacteria have previously been studied in laboratory. The treatments were, un-inoculated control with starter N and P; Rhizobium with half N and full P; Azospirillum with half N and full P; Pseudomonas with full N and half P; Rhizobium+ Azospirillum with half N and half P; Azospirillum + Pseudomonas with half N and half P; Rhizobium + Azospirillum + Pseudomonas with half N and half P; Full (recommended) N and P (100kg urea-N & 80kg P_2 O_{5-}P ha^{-1}). Recommended dose of K (@ 75kg $ha^{-1}$was applied to all the treatments. All the inoculated treatments except Rhizobium+ Azospirillum + ½ N + ½ P, produced significantly higher grain yield than un-inoculated control. Tripartite inoculation in the presence of half of recommended dose of N and P, brought 27% increase in grain yield over the uninoculated control, and it did not differ significantly from that where full dose of recommended N and P was given. The tripartite inoculation saved 50 % N and P without compromising on grain yield.

Key words. *Rhizobium, Azospirillum, Pseudomonas*, tripartite inoculants, wheat grain yield, grain N and P

INTRODUCTION

Escalating food and energy crisis has caused a shift in paradigm from chemical fertilizers alone to integrated plant nutrient management worldwide. Wheat is major food crop of Pakistan. Of the total cultivated area about 1.43 million hectare is under rainfed conditions, where benefits of chemical fertilizer are linked with timely rainfall. The farmers are resource poor. The highest achievable yield remains 2 tons/ha, which means 60% gap in achievable yield exists. Biofertilizers can be looked towards in such ecologies.

Root system is a unique micro site for the association of symbiotic and non-symbiotic N_2- fixers and phosphorus solubilizers and producers of bioactive compounds. There is growing evidences in favor of the beneficial effects of the application of *Azotobacter*, *Azospirillum* and Phosphorus solublizing bacteria (PSB) in providing plant nutrients to cereals and vegetables and supplementing the expensive inorganic fertilizer (Zaidi et al,2004). Improvement in crop yield and nutrient accumulation with mycorrhizal inoculation has been reported by various workers (Hammouda *et al*; 2001; Sahin, 2004). Mixture of rhizobacteria has been reported better for wheat by Freitas de, (2000). Biofertilizers could help to increase the availability of accumulated phosphate by solublization (Deubel and Merbach, 2005), efficiency of biological nitrogen fixation, inhibit denitrifiers and increase the availability of Fe, Zn etc, through production of plant growth promoting substances (Kucey *et al*; 1989). Trials with PSB indicate yield increase in rice (Tiwari, et, al 1989), maize (Khokhar *et al*., 2006), wheat (Hilali *et al*, 2001). Rhizobium previously well known as a symbiotic N fixer is also reported to play it's role as asymbiotic (associative & endophytic) diazotroph or plant growth promoter (Biswas *et al*., 2000 a, b) and has been reported (Narula et al, 2000) to produce nodules in the presence of certain chemicals/hormones. It has also been reported as phosphorus solubilizer (Khokhar et al, 2001). Role of dual or combined inoculation of N_2- fixing and phosphate solublizing bacteria have been recommended for providing more balanced nutrition for plants (Barea *et al*; 2005) Co-inoculation with beneficial bacteria increased yield in sorghum (Alagawadi & Gaur, 1992) barley (Belimove *et al*; 1995), soybean (Abd-Aallah & Omar, 2001) and wheat (Galal, 2003).

Hypothesis behind the present work was ascertaining supplemental role of indigenous formulation to expensive chemical fertilizers and thereby lower input cost for subsistent farmers in rainfed ecology.

MATERIALS AND METHODS

PAC-19/1 is a chickpea-nodulating, drought resistant rhizobium isolated from a loamy sand of Thal and has been well characterized (Khokhar and Khan, 1998; Khokhar et al, 2001). ZS1, was a nitrogen fixing *Azospirillum*, isolated from wheat rhizosphere from same ecology. Psm-na, a P-solubilizing *Pseudomonas* was isolated from wheat rhizosphere from Islamabad. Three microbes were grown on their respective media and inoculated to carrier as referred in Khokhar et al (2006).

Seed Germination test

Wheat (GA2002) seeds washed with tap water and surface sterilized with 17% Hydrogen peroxide were given seven inoculated treatments as mentioned previously by seed coating. One un-inoculated set of surface sterilized seeds was kept as control. The seeds were allowed to germinate in pre-sterilized Petri dishes, containing filter paper. Percent germination was recorded daily for a period of three days.

Field evaluation

The field trial with three test microorganisms *Rhizobium* PAC-19/1, *Azospirillum* (NFD-sg-1/94 / ZS1) and *Pseudomonas*(Psm-na) was conducted at experimental field (coarse loamy, hyperthermic Udic Ustochrept, NO3-N = 1.21%, Olsen-P = 5.5 mg kg^{-1}, K= 108 mg kg^{-1}, O.M= 1.1%, EC=0.5, pH=6.99) of National Agriculture Research Centre, Islamabad during winter (2006-2007). The land for the experiment was thoroughly prepared.

Pre-sowing soil samples were colleted from the depth of 0─15cm. Fertilizers applied were, urea -N @ 120kg/h, SSP –P_2O_5 @ 80kg/h and SOP-K_2O @ 75 $kg.h^{-1}$. The treatments were

1. un-inoculated control with 1/4N + 1/4P,
2. PAC-19/1+ ½ N + P
3. ZS1 + ½ N + P
4. Psm + N + ½ P
5. PAC-19/1+ ZS1+ ½ N + ½ P
6. ZS1 + Psm + ½ N + ½ P
7. PAC-19/1+ ZS1 + Psm + ½ N + ½ P
8. Recommended N+ P (100kg N & 80kg P_2O_5 ha^{-1}).

Potash was added @ 75$kg.h^{-1}$ to all treatments.

Micobes in each treatment were taken in ratios 1:1 or 1:1:1 (as the case may be) based on number of $cells.ml^{-1}$at late log phase, in carrier. Each treatment was replicated four times under randomized compete block design. Each replication consisted of eight rows in a 2x3m plot size. Fertilizer was added as per treatment at the time of plot preparation.

Seeds of wheat variety GA-2002 were coated with different inoculants as per treatments mentioned above, just before sowing. Experiment was maintained using standard cultural practices. The field soil had 35% moisture at sowing and rainfall during the crop season was recorded 407mm. Data on above ground biomass, root dry biomass, thousand grain weight and grain yield was recorded at maturity. Root and grain samples were dried at 70^{o}C and ground. Nitrogen content of each was estimated by micro-Kjeldahl method and phosphorus by acid digestion method (Winkleman et al, 1990)

The data obtained was subjected to the statistical analysis using STATISTICA software.

RESULTS AND DISCUSION

Seed germination

Wheat seed germination was 20% higher after 24 hrs on inoculation with *Azospirillum* or *Pseudomonas* alone while *Rhizobium* alone showed the same effect after 48hrs. Dual combinations of any of them also had similar effect. However coinoculation with three microbes together improved wheat seed germination by 40% when compared to uninoculated control (Table 2). Same tripartite combination improved seed germination in maize by 25% after 24h of inoculation (Khokhar et al, 2006). Effect on seed germination can be related with growth hormone production by the microbes. *Azospirillum* has been reported to produce IAA (Crozier *et al*; 1988) and promote seed germination in tomato and capsicum (Moya-Rodriguez *et al*; 2001). It has also been reported to affect maize as pre-germination treatment (Casanovas, 2003). The chickpea- *Rhizobium* has also been reported to produce IAA (Yasmin, 2003; Afzal & Bano, 2008). *Rhizobium* previously well known as a symbiotic N fixer is reported as (associative and endophytic) microorganisms in recent years (Biswas *et al*; 2000a, b). *Rhizobium* and *Pseudomonas* increased seed germination and yield in barley (Belimove *et al*; 1995), black gram (Tanwar *et al*; 2002) and wheat (Galal, 2003).

Growth and Yield

All inoculated treatments increased root dry biomass, number of tillers, above ground biomass and grain yield indices as compared to uninoculated control. Though inoculated treatments differed among themselves in different indices, yet were comparable to when full NPK applied.

Root dry weight

All inoculated treatments had significantly higher root dry biomass as compared to control. Highest root dry biomass was obtained when full NPK was supplied. Root dry biomass as produced by tripartite inoculant (*Rhizobium*+ *Azospirillum* +*Pseudomonas*) was not statistically different from that produced when full recommended NPK was applied. Maize roots showed similar response to same tripartite inoculants (Khokhar et al, 2006). Increased root weight with dual inoculation has been reported in rice (Yanni *et al* 1997) and in maize, (Chabot *et al* 1993) in barley (Hoflich *et al* 1994). Such results may be attributed to synergistic relationship of the inoculated microbes for improving root length and weight by producing growth activators. Higher root weight in inoculated than in uninoculated control (Table 3) reflects that inoculation positively affected root growth.

Number of tillers

Number of tillers was highest when full recommended Nitrogen and Phosphorus was applied. Number of tillers produced on inoculation of multimicrobe did not differ significantly from above while it saved 50% of N and P both. *Azospirillum*+ Psm-na ranked next at this fertilizer application rate. Increase in number of tillers has been reported by Hassanein and Goma (2001) in wheat when composite inoculum containing phosphate-solubilizing bacilli, *Azotobacter* and yeast was applied. Microbes multiply in rhizosphere during the first six weeks of plant growth and can show up their effect on tillering, an early yield component.

1000-grain weight

The 1000-grain weight of the wheat variety was affected by microbial inoculation (*Rhizobium Azospirillum,* and *Pseudomonas*). Highest 1000-grain weight (48.2g) was obtained when full NPK or Rhizobium+1/2N+P+K was applied. This was followed by when Psm-na +N+1/2P or tripartite inoculants+1/2N+1/2P were applied (Table 3). Same tripartite combination produced 1000-grain weight equal to NPK in Maize (Khokhar et al, 2006) also Thousands grain weight produced by Azospirillum+1/2N+P or Azospirillum+Pseudomonas+1/2N+1/2P were significantly lower than fertilized control. Previously positive effect of co-inoculation of *Azorhizobium* and *Azospirillum* on 1000-grain weight of wheat has been reported by El-Hawary *et al*, (1998). The present results indicate that microbes interaction in relation to metabolite transport needs to be studied more intensively.

Grain Yield

Grain yield showed good correlation (r= 0.766) with number of tillers. Increase in grain yield due to inoculation ranged from 8 – 27% over the uninoculated control. *Rhizobium* alone and in tripartite combination performed better than it did as dual inoculant with either of the two separately. Rhizobium alone seems to increase influx of metabolites into grains, in the presence of full P. Of all the inoculated treatments, highest increase (27%) in grain yield was recorded when all the three microbes, *Rhizobium*, *Azospirillum* and *Pseudomonas* were co-inoculated in the presence of half recommended dose of both N and P. Similar trends were reported when Maize cv. NARC3001 (Khokhar et al, 2006) was inoculated with same combinations of microbes, under rainfed conditions. The tripartite inoculants brought 50% increase in maize grain yield. Partition of metabolites seems to be affected differently by different inoculants. Similar reports have been made by Mehray et al (2009).

Table 1. Source and characteristics of bacterial isolates.

Isolate	Source	Salient characteristics
Rhizobium (PAC-19/1 /Sk-8/ Thal 8)	Chickpea nodules(Thal)	Chickpea-nodulating, motile, capable of growing equally well at 15°, 27° and 40°C. Acid production from mannitol.
Azospirillum (NFD-sg-1/94 / ZS1)	Wheat rhizosphere, (Thal)	ARA positive, motile spirilla, capable of growing equally well at 15°, 27° and 40°C. Resistant to Streptomycin and Ampicillin, 100μg/ml, pH reaction neutral
Pseudomonas (Psm-na)	Wheat rhizosphere, Islamabad	Gram-negative rods, white colonies on nutrient agar, pH reaction neutral, clearing zone formation on Pikovski's Agar medium.

Table 2. Effect of different bio-inoculants on wheat seed (GA 2002) germination (Percent).

Treatments	Day 1	Day 2	Day 3
Uninoculated control	40	60	60
PAC-19/1 (Sk-8/ Thal 8)	40	80	80
ZS1	60	80	80
Psm-qa	60	80	80
PAC-19/1+ ZS1	60	80	100
PAC-19/1+ Psm-na	60	80	100
ZS1+Psm-qa	60	80	100
PAC-19/1+ ZS1+ Psm-na	80	80	100

Table 3. Effect of different bio-inoculants on growth and yield of wheat (GA 2002) under rainfed conditions.

Treatments	Root dry biomass (g/m2)	Number of tillers/m2	Above ground dry biomass (kg/m2)	Grain yield (kg ha^{-1})	1000 grain Weight (g)
Uninoculated control+¼N + ¼P	26.7 c	213d	1.20 b	3620 d	35.1 b
PAC-19/1 (Sk-8/ Thal 8) + ½ N + P	38.1 b	263bc	1.40 ab	4790 ab	47.6 a
ZS1+ ½ N + P	40.5 b	233bcd	1.32 ab	3970 bcd	42.8 b
Psm-na+ N + ½ P	39.0 b	226cd	1.28 ab	3895 cd	47.2 ab
PAC-19/1+ ZS1+ ½ N + ½ P	41.4 b	246bcd	1.27 ab	3691 d	45.6 ab
ZS1+ Psm-na+½ N + ½ P	40.7 b	255bc	1.42 ab	3965 bcd	42.0 b
PAC-19/1+ZS1+Psm-na + ½ N + ½ P	43.0 ab	273ab	1.51 a	4624 ab	46.3 ab
NPK	49.5 a	313a	1.60 a	4896 a	48.2 a

Table 4. Effect of different bio-inoculants on nitrogen and phosphorus concentrations wheat (GA-2002).

Treatments	Root Nitrogen %	Seed Nitrogen %	Seed phosphorus %
Uninoculated control+¼N +¼ P	1.2 c	1.6 b	0.39 b
PAC-19/1 (Sk-8/ Thal 8) + ½ N + P	1.3 bc	1.7 b	0.52 a
ZS1+ ½ N + P	1.4 abc	1.7 b	0.50 a
Psm-na+ N + ½ P	1.3 bc	1.8 b	0.44 a
PAC-19/1+ ZS1+ ½ N + ½ P	1.2 c	1.8 b	0.42 a
ZS1+ Psm-na+½ N + ½ P	1.4 abc	1.7 b	0.41 a
PAC-19/1+ZS1+Psm-na + ½ N + ½ P	1.5 a	1.8 b	0.57 a
NPK	1.6 a	1.9a	0.60 a

Mixed microbial cultures have been reported to allow their components to interact with each other synergistically, thus, stimulating each other through physical or biochemical activities (Vassilev *et al*, 2001). Co-inoculation of wheat with *Rhizobium* + *Azospirillum* + *Pseudomonas* saved the cost of 50% N (urea) as well as 50% P (super phosphate). Same inoculants saved 50% cost of fertilizer in maize (Khokhar et al, 2006). Previously 40%

saving on N-fertilizer has been reported by Santa *et al* (2004) by inoculating maize CV Carqil-909 in Brazil, while 50% saving due to *Azorhizobium* and *Azospirillum* has been reported by (Woodard and Bly 2000), for the same crop. Trans Van et al (2000) reported increase in grain yield of rice equivalent to 25-30kg of N fertilizer, in response to *Burkholderia vietnamiensison* inoculation in acid soils of Vietnam . The present results indicate that wheat GA2002 also supported microbe-plant interaction, because signal molecules from plants have been reported to influence microbe-soil interaction. (Singh *et al*; 2004; Barea *et al* 2005).It also prove that multimicrobe inoculant performed best among all inoculated treatments and was at par with full fertilized treatment.

Nitrogen and Phosphorus content (%)

Root nitrogen (%) showed variation in different inoculated treatments. Highest root %N (which equaled to when full NPK was applied) was recorded when all three microbes were coinoculated. Azospirillum alone accumulated N in root which did not differ significantly from tripartite inoculant

Highest percentage grain nitrogen (1.14%) and phosphorus (0.60%) of grain was recorded with NPK (full recommended dose). Of all the inoculated treatments, *Rhizobium* in combination with *Azospirillum* and *Pseudomonas* (in the presence of half dose of nitrogen and phosphorus) was at par with NPK in terms of root nitrogen and grain phosphorus (Table 4).

Higher %N of grain due to *Pseudomonas* alone may be attributed to improved uptake of N from soil as a result of increased P-availability. Previously available soil-P has been reported to improve N-uptake by plant root (Kucey et al, 1989). Lesser percentage of grain nitrogen and higher root N on application of tripartite inoculant may be attributed to lowered mobilization of N from root to grain.

Large variation in root N as compared to grain N indicates diverse type of behaviour of different inoculants. Different strains of rhizobia have been reported to affect transport of metabolites differently. However taking into account the grain yield/h, tripartite inoculant accumulated highest amount of nitrogen and phosphorus in grains just next to NPK.

Highest grain phosphorus (0.57%) was recorded due to inoculation of tripartite inoculant in the presence of nitrogen @ 60 kg/ha and phosphorus @ 40 kg/h and was non-significantly different from when full dose of N+P was applied. Dual inoculation with half dose of N+P or N or P did not differ significantly from single inoculation with half dose of N +P in grain percent Phosphorus. Increased seed P content by phosphate solublizing microorganism alone has been reported by Mehana & Wahid (2002). Higher N, P and K content of wheat grain has been reported (Mehray et al, 2009) due to coinoculation of *Rhizobium* and *Azospirillum brasilense.*

It can thus be concluded that best overall performance was shown by multi-microbe inoculant as it increased seed germination by 40% and grain yield by 27%, Percent Phosphorus of grain was also highest when tripartite inoculant was used. Co-inoculation of wheat with *Rhizobium* + *Azospirillum* + *Pseudomonas* in combination with 50% N+P produced root dry biomass, above ground biomass and grain yield, not significantly different from when full recommended dose of N and P was applied. Its use can thus save 50% cost of urea as well as of SSP. The tripartite inoculant can thus be used as biofertilizer in low fertility soil in low rainfall area for the profit of resource poor farmer.

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