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Azotobacter and *Azospirillum* Inoculants as Biofertilizers in Canola (*Brassica napus* L.) Cultivation

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Abstract: In order to evaluate the effect of additional application of *Azotobacter* and *Azospirillum* inoculants (Biofertilizers) on canola (*Brassica napus* L.) yield and profitability, a split-plot experimental design with 20 treatments was carried out during 2004-2005 with four replications in the North of Iran. High yielding canola (cv. Hyola 401 hybrid), was grown in rotation after wheat. Two levels of biofertilizers as control and seeds inoculation in main plot and 10 treatments of chemical fertilizers comprising N, P, K and their combinations, NPKS and NPK Zn in sub plots were applied. The treatment T_{20} resulted in the maximum seed yield (3374 kg ha^{-1}) coinciding with the maximum number of pods per plant (246 pods per plant) followed by the treatments T_{19}, T_{18} and T_{15}. Out of these 4 treatments, it was discovered that the highest net benefit of adding biofertilizers was observed at T_{15} (1.07 million rials.ha^{-1} = 117.7 \$.ha^{-1}). The research projects how the efficiency of these biofertilizers was maximum in presence of N and P fertilizers, while in the presence of K and Zn fertilizers at T_{20} it resulted mainly in the increase of fodder rather than seed. The seed N, protein and the oil percentage remained unaffected by biofertilizers application.

Key words: *Azotobacter*, *Azospirillum*, biofertilizers efficiency, canola, profitability

INTRODUCTION

Canola (*Brassica napus* L.) growing has become attractive to the farmers in Iran, as an indigenous source of vegetable oil and animal meal. A healthy canola plant is considered as nitrogen demanding commercial crop and it has been shown that an addition of 135 kg ha^{-1} nitrogen would significantly increase the canola yield. Although the application of nitrogen fertilizer along with the others chemical fertilizers have proved to be essential but their higher application may result in environmental disasters like NO^{-}_{3} pollution of ground water, soil acidification and increased denitrification resulting in higher emission of N_2O to the atmosphere, which may impact global warming. These problems have renewed public interest in exploring alternate or supplementary non-polluting sources of N for agriculture. Finding an alternative for such a nutrient has become important. Soil microorganisms like *Azotobacter* and *Azospirillium* are free living N_2 fixing bacteria which can successfully grown in the rhizospheric zone of crops and fix 10-20 kg N ha^{-1} cropping season. Besides N_2 fixation these bacteria synthesize and secret considerable amounts of biologically active substances like gluconic acid and the ability of direct phosphate solubilization which enhance root growth of plants (Rodriguez *et al*., 2004). *Azotobacter* along with other N_2 fixing bacteria like rhizobium play important role in yield-attributing characters owing to the production of siderophores which regulates the availability of nutrients to the crop (Boiero *et al*., 2007).

Rock *et al*. (1996) examined *Rhizobium leguminosarum* bv. *phaseoli* and *Pseudomonas* strains for their plant growth-promoting potential on lettuce and forage maize. The plants were grown in field conditions in the site having very fertile soil tended to increase the dry matter yield of lettuce shoots ($p\leq 0.10$). Lettuce inoculated with rhizobia had a 6% higher P concentration ($p\leq 0.10$) than the uninoculated control. In moderately fertile soil the dry matter of maize shoots was significantly increased ($p\leq 0.05$) by inoculation with strain 24 plus 17.5 kg ha^{-1} P-superphosphate, or with strain P31 plus 35 kg ha^{-1} P-superphosphate. Inoculation with PSM did not affect lettuce P uptake in the less fertile soil but in the moderately fertile soil, maize plants inoculated had 8% higher P concentration than the uninoculated control ($p\leq 0.01$). They have concluded that rhizobia function as plant growth promoting rhizobacteria with the nonlegumes lettuce and maize.

Shukla *et al*. (2002) reported that application of *Azotobacter* resulted significantly in higher number of

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seeds/siliquae, TDM, branches/plant and the length of siliquae in Indian mustard. Sharma *et al.* (1997) reported that the oil content of Indian mustard decreased by successive increasing in N level and application of *Azotobacter*, suggesting that oil and protein production increased significantly when nitrogen applied either through *Azotobacter* or urea. Cecilia *et al.* (2004) reported wheat grains harvested from *Azospirillium*-inoculated plants contained significantly higher Mg, K and Ca than non-inoculated plants. They also proved that grain yield loss to drought was 26.5 and 14.1% in non-inoculated and *Azospirillium*-inoculated plants respectively. Khalid *et al.* (2004) reported that Peat-based seed inoculation with selected PGPR isolates exhibited stimulatory effects on grain yields of tested wheat cv. in pots (up to 14.7% increase over control) and field experiments (up to 27.5% increase over control).

Not much experimental work has been conducted on the use of such N_2 fixing bacteria on the growth and yield of canola. The only attempts made on canola refer to the application of inoculation with *Penicillium bilaji*, *Bacillus thuringiensis* and Phosphate solubilizing *Rhizobacteria* for the P-uptake, vegetative growth and grain yield of canola (Freitas *et al.*, 1997). It seems that finding an alternative factor to reducing the harmful effects of nitrogen exceed application in the environment as well as keeping the production level for crops is essential, therefore the study of the effects of Biofertilizers (*Azotobacter* and *Azospirillium*) in the growth and productivity of canola have been conducted.

MATERIALS AND METHODS

A split-plot experimental design with 20 treatments and 4 replications was carried out during 2004-2005 cropping season. The canola (cv. Hyola 401 hybrid), a high yielding early maturity canola hybrid, was grown during the months October to May, which is generally a humid season in Northern Iran. The area receives an average of 700-800 mm rain fall and has a relative humidity of 77%. Bacterial strains of *Azospirillum* and *Azotobacter* inoculants were applied in the main plots and a combination of chemical fertilizers, both macro and micronutrients were applied in the sub plots. The experiment was carried out at the Baiecola Agricultural Research Station in Mazandaran province (Iran).

The strains of *Azotobacter* and *Azospirillum* were isolated from the different samples of the soils of local area. To facilitate the identification of *Azotobacter* spp., modified mannitol agar medium with 10 g of glucose mannitol per liter as a carbon source was used. For *Azospirillum* NFb a potato extract media was used. The isolates, thereafter were compared with the reference strains. Combined inoculants of *Azotobacter chroococcum, Azospirillum lipoferum* and *Azospirillum brasilense* strains were applied for the biofertilizer treatments.

The canola crop was taken as a second crop in rotation after wheat. It was grown under rain fed conditions. Soil samples were collected and analyzed to know the composition or the nutrients availability and the crop requirements for the nutrients. The chemical fertilizers were chosen and applied accordingly. The experimental soil was texturally silt-clay, with pH 7.6, 1.3% OC, 180 ppm of available K, 7 ppm of available P, 18 ppm Mn, 10 ppm Fe, 1.1 ppm B and 0.96 ppm of Zn. The chemical fertilizers consisting N, P, K, S and Zn were applied prior to cultivation, except the nitrogen fertilizer which was applied in split stages, once basal and twice top-dressed.

The experimental field was divided into 4 blocks. Each block was then divided into 20 plots in all the experiment was done in 80 plots of each of 10 m^2 area. For calculating the seed oil, protein and N content number of seed were sampled separately from each treatment, the seed oil and protein content were measured following Nuclear Magnetic Resonance Spectrophotometry (NMR) and Micro Kjeldahl digestion using automated colorimetric analysis, respectively. Data were analyzed following the analysis of variance technique (ANOVA) and then the mean differences were adjudged by Duncan's Multiple Range Tests (DMRT).

RESULTS AND DISCUSSION

Yield

Application of chemical fertilizers on yield (Treatments T_2 to T_{10}): The effect of chemical fertilizers on yield was statistically significant ($p<0.01$). The application of N and P fertilizers at treatments T_2 and T_3 did enhance the yield by 148 and 133% over the control (Table 1). Favorable reports exist on canola seed yield by application of N fertilizer (Ozer, 2003; Hocking *et al.*, 2003) and P being a structural component of nucleic acid and protein and nucleoprotein its application does favor significantly the seed yield, LAI and TDM in canola and other *brassica* species (Lickfett *et al.*, 1999).

The present experiment indicated that the yield can be augmented (Table 1) substantially, above 3000 kg ha^{-1} by a mixture of NPKZn (T_{10}) and NPKS (T_9) but applying NPK alone (T_8) the yield remained below that level. The supplementary addition of Zn and S increased the yield marginally, but these three treatments show the same statistical rank. In certain soils the application of S alone or in combination with NPK has also favored substantially the increase in canola yield (Jayan and Patro, 1999; Hocking and Strapper, 2001; Santonoceto *et al*., 2002).

Application of biofertilizers (treatments T_{12} to T_{20}): Application of biofertilizers alone had little effect on the yield (T_{11}) but when applied in conjunction with N (T_{12}) and P (T_{13}) or with NP (T_{15}) it made a tremendous difference in the number of pods per plant that is reflected in the seed yield (Table 1). On the other hand, the application of biofertilizers with K (T_{14}) and with PK (T_{17}) had only little effect on the number of pods per plant as also on the seed yield. The yield obtained was even lower than when N (T_{12}) and P (T_{13}) fertilizers were applied individually with biofertilizers. The number of pods per plant and the yield obtained at T_{15} far exceeded those obtained at T_{16} and T_{17} where biofertilizers were applied with NK and PK fertilizers. Thus the efficiency of biofertilizers in the presence of K fertilizer seems to get subdued, which is also seen at T_{18}. The seed yield obtained at T_{19} (BF+NPKS) and T_{20} (BF+NPK Zn) exceeded that at T_{15}, reflected also in the increase in the number of pods per plant.

Positive reports of application of biofertilizers (*Azotobacter* and *Azospirillum* and other bacteri) on yield are available on crops like: Indian mustard (Suneja and Lakshminaraya, 2001), cotton (Yue *et al*., 2007), corn (Albrecht *et al*., 1981), sorghum (Singh *et al*., 2005) wheat (Cecilia *et al*., 2004), tobacco (Li *et al*., 2007) and barley (Ozturk *et al*., 2003), which is attributed to the enhancement of factors like N_2 fixation nitrate reductase activity, intake of NO_3, NH_4, H_2PO_4, K and Fe, plant water status and production of phytohormones such as Indol acetic acid (Wani *et al*., 1998; Antoun *et al*., 1998; Arshad and Frankenberger, 1998). It is possible that the enhancement of such factors has been instrumental in the present experiment also, especially at treatments T_{15}, T_{19} and T_{20}.

Economics: The expenditure incurred per ha for applying different chemical fertilizers varied (Table 2) and becomes costlier every year, but the additional cost of biofertilizers is only marginal. The selling price per kg, as fixed by the Government of Iran, though increases every year, is not sufficient enough to sustain. The yield obtained at most of the treatments exceeds the average canola yield in the world (1750 kg ha^{-1}) and in Iran (1640 kg ha^{-1}), the role played by biofertilizers is visible in all treatments T_{12} to T_{20}. For achieving the yield comparable to the average level of canola yield in Germany (4133 kg ha^{-1}) and France (3540 kg ha^{-1}), the application of biofertilizers in conjunction with N, P and Zn seems to be the only alternative.

Table 1: Effect of chemical and biofertilizers on yield, pods per plant and cost of fertilizers

Treatments	Seed yield (kg ha^{-1})	Pods/plant	Total cost of fertilizers million rials*
T_1 = Control	736.313j	75I	
T_2 = N	1827.438fg	137f	0.11
T_3 = P	1718.938fg	125fg	0.08
T_4 = K	1266.125hi	94hi	0.08
T_5 = NP	2621.537cd	186de	0.19
T_6 = NK	1936.250ef	141f	0.19
T_7 = PK	1520.075gh	113gh	0.16
T_8 = NPK	2997.050abc	211bc	0.27
T_9 = NPKS	3095.100ab	224abc	0.27
T_{10} = NPKZn	3141.25ab	230ab	0.28
T_{11} = BF	916.100ij	87I	0.01
T_{12} = BF+N	2409.250d	176e	0.12
T_{13} = BF+P	2303.037de	165e	0.08
T_{14} = BF+K	1662.025fg	121fg	0.09
T_{15} = BF+NP	2910.862bc	206cd	0.19
T_{16} = BF+NK	2318.900de	165e	0.20
T_{17} = BF+PK	1942.863ef	141f	0.16
T_{18} = BF+NPK	3041.050ab	214bc	0.28
T_{19} = BF+NPKS	3282.162ab	240a	0.28
T_{20} = BF+NPKZn	3374.162a	246a	0.28
LSD (0.01)	1044.00	21.39	

BF = Biofertilizers; alphabets represent statistical similarities
*1 m.Rials = 110 $

Table 2: Monitory benefits (million Rials) at different treatments

Treatments	Sale value (million Rials)*	Benefit (million Rials)	Benefit of adding BF (million Rials)
T_1 = Control	2.72	2.72	
T_2 = N	6.76	6.65	
T_3 = P	6.36	6.28	
T_4 = K	4.68	4.60	
T_5 = NP	9.70	9.51**	
T_6 = NK	7.16	6.97	
T_7 = PK	5.62	5.46	
T_8 = NPK	11.09	10.82	
T_9 = NPKS	11.45	11.18	
T_{10} = NPKZn	11.62	11.34	
T_{11} = BF	3.39	3.38	0.66
T_{12} = BF+N	8.91	8.79	2.14
T_{13} = BF+P	8.52	8.44	2.16
T_{14} = BF+K	6.15	6.06	1.46
T_{15} = BF+NP	10.77	10.58***	1.07
T_{16} = BF+NK	8.58	8.38	1.41
T_{17} = BF+PK	7.19	7.03	1.57
T_{18} = BF+NPK	11.25	10.97	0.15
T_{19} = BF+NPKS	12.14	11.86	0.68
T_{20} = BF+NPKZn	12.48	12.20	0.86

*1 million Rials = 110 $, **Taking 9.5 million Rials as the cut off value for recognizing profitable treatment by using chemical fertilizers, ***10.5 million Rials as the cut off value for treatments using additional biofertilizers

If the benefit is calculated by subtracting the investment cost from the sale value it is maximum at T_{20} and considering 9.5 million rials.ha^{-1} as the cut off value one may recognize T_5, T_8, T_9 and T_{10} as profitable treatments with application of only chemical fertilizers. The cut off value for treatments using additional biofertilizers would be above 10.5 million rials.ha^{-1} (Table 2). Considering that the treatments T_{15}, T_{18}, T_{19} and T_{20} are profitable. If the benefit is viewed in terms of improvement in yield (Table 1), consequent to adding biofertilizers, the results obtained at T_{15} could be adjudged better than those at T_{18}, T_{19} and T_{20}.

The efficiency of application of biofertilizers towards nutrient uptake by canola plant is betrayed in the improvement of important morphological and physiological characteristics (Table 3, 4) as well, at treatments T_{15} and T_{20}.

CONCLUSION

Comparing the results obtained at T_{15} (Biofertilizers + NP) and T_{20} (Biofertilizers + NPK and Zn), it is observed that (Table 3, 4) application of biofertilizers was more effective at T_{15} for seed yield augmentation and oil yield/ha. The seed yield increased from 2622 kg ha^{-1} at T_5 (NP) to 2911 kg ha^{-1} at T_{15}. At T_5 it already showed an increase of 256% over the control (T_1), but at T_{15} the increase was 217% over T_{11} and 295% over T_1. Though the highest seed yield (3374 kg ha^{-1}) was obtained at T_{20}, the improvement over T_{10} (NPK Zn) was lesser than that at T_{15} over T_5. The treatment T_{10} already showed an increase of 319% over T_1, but the addition of biofertilizers at T_{20} resulted in an increase of 268% over T_{11} and 358% over T_1.

The increase in the yield at T_{15} and T_{20} was consequent to the greater proliferation of the number pods per plant. At T_{15} the number of pods per plant (196) showed an increase of 9.49% over T_5 and 117% over T_{11}. At T_5 the number of pods per plant already showed an increase of 138% over control (T_1). At treatment T_{20} the number of pods per plant (249) showed an increase of 8.26% over T_{10} and 176% over T_{11}. At T_{10} also an increase of 206 % was observed over control T_1. Therefore it is concluded that the increase in the number of pods per plant at T_{15} over T_5 (9.49%) was more than the increase at T_{20} over T_{10} (8.26%). Among the four useful treatments T_{15}, T_{18}, T_{19} and T_{20}, though the minimum number of pods was obtained at T_{15} (196), the percent increase in the number of pods by using additional biofertilizers was maximum. The achievement of 196 pods per plant was concluded to be essential for obtaining the seed yield beyond 2900 kg ha^{-1}, which is almost doubled the average seed yield of the Mazandaran Province.

The application of biofertilizers decreased substantially the Harvest Index, which is the ratio of economical yield (seed yield) to total plant biological yield including seed yield also at both the T_{15} (22.46%) and T_{20} (20.56%) when compared with that at T_5 (22.86%) and T_{10} (22.87%), respectively. But the percentage of lowering the HI at T_{15} was less than that at T_{20}. The higher HI indicates a corresponding increase in seed yield rather than stover, which seems to have been promoted more at T_{20}. While the increase in HI at T_5 and T_{10} over control (T_1) was almost similar the biofertilizers inoculation promoted the seed yield at T_{15} compared to T_{20}. The TDM at T_{20} showed a remarkable increase compared to only a marginal increase at T_{15} (Table 3, 4).

Table 3: Plant yield and yield attributing characters at pair T_{10}/T_{20}.

Treatments	T_{10} = NPKZn	T_{20} = BF +NPKZn	Increase of T_{20} over 10 (%)
Seed yield (kg ha^{-1})	3141.30	3374.20	7.41
Pods/plant	230.00	246.00	6.95
Plant height (cm)	120.55	128.28	6.41
1000 seed wt. (g)	4.35	4.38	0.68
Seed N (%)	3.84	3.92	2.08
Harvest index (%)	24.21	22.47	-7.18
Oil content (%)	45.83	46.34	1.11
Oil yield (kg ha^{-1})	1439.00	1563.00	8.61
Protein content (%)	24.02	24.90	3.66
LAI at flowering	4.26	4.75	11.50
TDM (kg ha^{-1})	9830.00	11640.00	18.41

Table 4: Plant yield and yield attributing characters at pair T_5/T_{15}.

Treatments	T_5 = NP	T_{15} = BF +Npover	Increase of T_{15} 5 (%)
Seed yield (kg ha^{-1})	2621.5	2910.9	11.03
Pods/plant	186	206	10.75
Plant height (cm)	112.8	120.0	6.38
1000 seed wt. (g)	4.20	4.28	1.90
Seed N (%)	3.81	3.92	2.88
Harvest index (%)	22.94	24.62	7.32
Oil content (%)	45.42	45.52	0.22
Oil yield (kg ha^{-1})	1191	1326	11.33
Protein content (%)	23.82	24.52	2.93
LAI at flowering	3.9	4.5	15.38
TDM (kg ha^{-1})	8787	8890	1.17

The plant height is an important contributing factor towards higher TDM. Among the treatments T_{15}, T_{18}, T_{19} and T_{20} the maximum plant height was achieved at T_{20} (135 cm), which coincided with the maximum TDM (11940 kg ha^{-1}). At T_{15} the plant height was 118 cm, which is minimum among these four treatments. Obtaining the seed yield beyond 2900 kg ha^{-1} required to having taller plants ($\geq$118 cm), which can be considered as a bio-indicator for having a healthier plant growth and high seed yield. However plant height alone cannot be a sufficient indicator for expecting a high yield, the application of NP fertilizers together with biofertilizers is essential.

The maximum seed oil content (46.11%) was obtained at T_{20}, which showed an increase of 0.61% over T_{10}. At T_{15} however, the seed oil percent (44.88%) is lesser than at T_{20}, showing an increase of 0.51% over the respective treatment of T_5. When the oil yield is calculated it showed a substantial increase of 11.54% from 1170 kg ha^{-1} at T_5 to 1350 kg ha^{-1} at T_{15}. At T_{20} although the maximum oil yield (1556 kg ha^{-1}) was obtained, the percent increase in oil yield over that at T_{10} was only 9.96%.

Seed protein content is useful for animal feed after oil extraction and is depended on the seed-N contents. The maximum seed-N (4.12%) and protein content (25.75%) was obtained at T_{15}. The percent increase in seed-N at T_{15} over T_5 (7.02%) was more than that at T_{20} over T_{10} (5.61%). Similarly the percent increase in seed protein content at T_{15} over T_5 (7.02%) was more than at T_{20} over T_{10} (5.16%).

Considering the above discussion it is concluded that using combined inoculants of *Azotobacter chroococcum*, *Azospirillum lipoferum* and *Azospirillum brasilense* as biofertilizers in conjunction with NP fertilizers at T_{15} was more profitable than using such biofertilizers along with NPK Zn together at T_{20} in enhancement of canola seed yield.

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