Plant and Soil **265:** 123–129, 2004.

Sugar beet and barley yields in relation to inoculation with N_2-fixing and phosphate solubilizing bacteria

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Received 11 October 2003. Accepted in revised form 3 February 2004

Key words: nitrogen fixation, phosphate solubilization, dual inoculation, biofertilizers, *Bacillus* ssp., sugar beet, barley

Abstract

Recently, there has been a resurgence of interest in bioorganic fertilizers as part of sustainable agricultural practices to alleviate drawbacks of intensive farming practices. N_2-fixing and P-solubilizing bacteria are important in plant nutrition increasing N and P uptake by the plants, and playing a significant role as plant growth-promoting rhizobacteria in the biofertilization of crops. A study was conducted in order to investigate the effects of two N_2-fixing (OSU-140 and OSU-142) and a strain of P-solubilizing bacteria (M-13) in single, dual and three strains combinations on sugar beet and barley yields under field conditions in 2001 and 2002. The treatments included: (1) Control (no inoculation and fertilizer), (2) *Bacillus* OSU-140, (3) *Bacillus* OSU-142, (4) *Bacillus* M-13, (5) OSU-140 + OSU-142, (6) OSU-140 + M-13, (7) OSU-142 + M-13, (8) OSU-140 + OSU-142 + M-13, (9) N, (10) NP. N and NP plots were fertilized with 120 kg N ha^{-1} and 120 kg N ha^{-1} + 90 kg P ha^{-} for sugar beet and 80 kg N ha^{-1} and 80 kg N ha^{-1} + 60 kg P ha^{-1} for barley. The experiments were conducted in a randomized block design with five replicates. All inoculations and fertilizer applications significantly increased leaf, root and sugar yield of sugar beet and grain and biomass yields of barley over the control. Single inoculations with N_2-fixing bacteria increased sugar beet root and barley yields by 5.6–11.0% depending on the species while P-solubilizing bacteria alone gave yield increases by 5.5–7.5% compared to control. Dual inoculation and mixture of three bacteria gave increases by 7.7–12.7% over control as compared with 20.7–25.9% yield increases by NP application. Mixture of all three strains, dual inoculation of N_2-fixing OSU-142 and P-solubilizing M-13, and/or dual inoculation N_2-fixing bacteria significantly increased root and sugar yields of sugar beet, compared with single inoculations with OSU-140 or M-13. Dual inoculation of N_2-fixing *Bacillus* OSU-140 and OSU-142, and/or mixed inoculations with three bacteria significantly increased grain yield of barley compared with single inoculations of OSU-142 and M-13. In contrast with other combinations, dual inoculation of N_2-fixing OSU-140 and P-solubilizing M-13 did not always significantly increase leaf, root and sugar yield of sugar beet, grain and biomass yield of barley compared to single applications both with N_2-fixing bacteria. The beneficial effects of the bacteria on plant growth varied significantly depending on environmental conditions, bacterial strains, and plant and soil conditions.

Introduction

Nitrogen and phosphorus are known to be essential nutrients for plant growth and development. The global nitrogen cycle pollutes groundwater and increases risk of chemical spills. The production of chemical fertilizers is a highly energy-intensive process using large amounts of fossil energy. High- input farming practices achieving high yields have created environmental problems and degradation in natural resources. Large

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quantities of chemical fertilizes are used to replenish soil N and P, resulting in high costs and severe environmental contamination. Consequently, there has recently been a growing level of interest in sustainable agricultural practices to alleviate detrimental effects of intensive farming currently practiced. Increasing and extending the role of biofertilizers would reduce the need for chemical fertilizers and decrease adverse environmental effects. Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers.

Rhizosphere associated N_2-fixing and P-solubilizing bacteria have increasingly been used in non-legume crop species such as sugar beet, sugar cane, rice, maize and wheat (Döbereiner, 1997; Hecht-Buchholz, 1998; Schilling et al., 1998). Trials with *Bacillus* species indicated yield increases in rice (Tiwari et al., 1989), cereals (Belimov et al., 1995; Çakmakçi et al., 2001; Öztürk et al., 2003) and maize (Pal, 1999). Asymbiotic N_2-fixing bacteria were reported to replace 60% of N requirements of sugar cane amounting to 200 kg N/ha^{-1} (Döbereiner et al., 1993; Urquiaga et al., 1992). *Bacillus* species used as biofertilizers may have direct effects on plant growth through the synthesis of plant growth hormones (Amer and Utkheda, 2000; Hecht- Buchholz, 1998), N_2-fixation (Yoneyama et al., 1997) and synthesis of the enzymes modulating the level of plant growth promoting rhizobacteria (Kumar and Narula, 1999).

Some of the above bacteria may also solubilize inorganic phosphate, making soil phosphorus otherwise remaining fixed available to the plants (Kumar and Narula, 1999; Whitelaw, 2000) due to excretion of organic acids (Kucey et al., 1989; Whitelaw, 2000) and through carbon and nitrogen sources, salt, pH, temperature (Nautiyal et al., 2000). Phosphate solubilizing *Bacillus* spp. stimulates plant growth through P nutrition (Whitelaw et al., 1997), increasing the uptake of N, P, K and Fe (Biswas et al., 2000). Phosphorus biofertilizers could help increase the availability of accumulated phosphates for plant growth by solubilization, enhancing plant growth by the increasing the efficiency of biological nitrogen fixation and the availability of Fe, Zn through production of plant growth promoting substances (Kucey et al., 1989).

Combined inoculations with N_2-fixing and P-solubilizing bacteria were more effective than single microorganisms controlling soil-borne pathogens (Fukui et al., 1994) and providing a more balanced nutrition for plants (Belimov et al., 1995). Dual inoculations increased yields in sorghum (Alagawadi and Gaur, 1992) and barley (Belimow et al., 1995) compared to single inoculations with N_2-fixing or P-solubilizing bacteria. Therefore, a study was conducted in order to investigate the effect of single, dual and multiple inoculations with N_2-fixing and P-solubilizing bacterial species on sugar beet and barley yields further to field trials with their single applications (Çakmakçi et al., 2001).

Materials and methods

An experiment was carried out on the Experimental Farm of the Turkish Sugar Factories Corporation in Erzurum in Eastern Anatolia (29°55′ N and 41°16′ E with an altitude of 1950 m). In order to investigate the effects of single, dual and mixed inoculations with N_2-fixing bacteria (*Bacillus* OSU-140 and *Bacillus* OSU-142) and a strain of phosphorus solubilizing *Bacillus* (M-13) on yield and yield components of sugar beet (*Beta vulgaris* cv. Loretta) and barley (*Hordeum vulgare* cv. Tokak) under field conditions in 2001 and 2002.

The bacterial strains, *Bacillus* OSU-140, *Bacillus* OSU-142 were originally isolated from tomato plants at the Ohio State University (USA), and M-13 was isolated from pepper plants at Atatürk University, Erzurum, Turkey. They were investigated and selected for their antifungal and antibacterial properties in the previous studies (Eşitken et al., 2002; Şahin and Miller, 1999). OSU-140 and OSU-142 were the most effective N_2-fixing bacteria in the previous field experiments with sugar beet and barley (Çakmakçi et al., 2001). In the present study, P-solubilizing ability of M-13 was demonstrated based on the qualitative and quantitative methods described previously (Mehta and Nautiyal, 2001) under the laboratory conditions (Table 1).

The bacterial strains were characterized by morphological, biochemical and physiological tests including pigment production on nutrient agar medium, the gram (KOH) reaction, catalase, oxidase, starch hydrolysis, nitrate reduction activities and growth at 36 °C on N-free basal medium (Forbes et al., 1998). The bacterial strains were maintained for long-term storage in nutrient broth with 15% glycerol at −80 °C for further tests. For this experiment, pure cultures were grown in nutrient broth (NB) at 28 °C and diluted to a final concentration of 10^9 cfu ml^{-1} in sterile distilled water containing 0.025% Tween 20. Seeds were then treated with the bacterial suspensions for

Table 1. Sources and biochemical characteristics of the bacterial strains tested

Bacterial Strain	Sources	Biochemical Characteristics								
		Gram stain	Phosphate solubilization	Catalase	Oxidase	Pigment	Nitrate reduction	Starch hydrolysis	Growth at 36 °C	Growth in N-free basal medium
Bacillus OSU-140	tomato	+	−	+	−	−	+	+	+	+
Bacillus OSU-142	tomato	+	−	+	−	−	+	+	+	+
Bacillus M-13	pepper	+	+	+	−	−	+	+	+	+

30 min. Seeds surface-sterilized by soaking in 25% commercial–grade bleach (sodium hypochlorite) for 5 min, followed by thorough washing under running tap water and air-drying aseptically overnight at room temperature were soaked into the bacterial suspension for an hour in a shaker of 150 rpm before sowing.

The experimental soil was a clay loam with organic matter content of 2.5% and with 2.7% free carbonate (pH = 7.8). Available P_2O_5 (Olsen et al., 1954) and K_2O contents were 51 and 1328 kg ha^{-1}, respectively. With an average temperature and total rainfall of 4.2 °C and 401 mm, plant growth is restricted to the period between May and October in the region. Winter wheat (2001) and spring barley (2002) was preceding crop for sugar beet while barley followed sugar beet in both years.

The experimental design consisted of five completely randomized blocks in a factorial arrangement each having 10 main treatments as (1) Control (without inoculation and any fertilizer application), (2) *Bacillus* OSU-140, (3) *Bacillus* OSU-142, (4) *Bacillus* M-13, (5) (OSU-140 + OSU-142), (6) (OSU-140 + M-13), (7) (OSU-142 + M-13), (8) (OSU-140 + OSU-142 + M-13), (9) Nitrogen, and (10) Nitrogen + Phosphorus. Sugar beet received 120 kg N ha^{-1} in (N) plots and 120 kg N ha^{-1} + 90 kg P ha^{-1} in NP plots in the form of urea and triple super phosphate applied during disk-harrowing in spring and before the first hoeing in equal amounts (N) or during deep ploughing in autumn (P). Barley received 80 kg N ha^{-1} (N) and 80 kg N ha^{-1} + 60 kg P ha^{-1} in (NP) plots. N fertilizer was applied in two equal parts in spring and at tillering stage while the whole of P fertilizer was applied during soil preparation in spring. All plots in both trials received farmyard manure at 30 t ha^{-1} before ploughing in autumn.

Sugar beet seeds were sown with a plot drill in 8 × 2.25 m plots so as to give 45 cm inter and 8 cm intra row spacing on 2 May both in 2001 and 2002 following the bacterial inoculation of seeds depending on the treatment. Barley was sown in 7 × 1.2 m plots having 6 rows so as to give 400 seeds per m^{-2} on 24 April in both years. Due care was taken not to contaminate and mix bacterial inoculation during sowing.

When sugar beet seedling reached 2–6 leaf stage, thinning to 20 cm intra-row plant spaces was performed. Plant density was adjusted to 9.6 plants m^{-2} after thinning all plots (Çakmakçi et al., 1998). Plots received five irrigations starting from mid June. No pesticide was applied. Harvesting was done on the 15th of October in both year's excluding 1 side row and 1 m from each end of plots. Data were made on root yield and analysis of sugar content, a-amino N, Na and K content were carried out in laboratories of The Sugar Institute of Turkish Sugar Factories Corporation. White sugar content (WSC) and white sugar yield (WSY) were calculated as WSC = SC − [0.343 (Na + K) + 0.094 a-amino N + 0.29] and WSY = (WSC/100) × root yield (t/ha) (Reinefeld et al., 1974).

Barley was irrigated twice at the beginning of stem elongation and booting stage. Weeding was done by hand when required. Harvesting was performed excluding side rows and 1 m from each end of plots on 1st of August 2001 and 12th of August 2002. Plants were cut by hand, approximately 5 cm above ground level to measure grain and stubble yields.

The data made from both trials were subjected to analysis of variance using MSTATC Statistical Package and means were separated according to Duncan Multiple Range Test. The chi-square test (Gomez and Gomez, 1984) was used to verify homogeneity of variance before combining data. Error means squares from each year for a trait were homogeneous. Analysis was also carried out among the parameters investigated on the basis of two year's data.

Table 2. Combined analysis of yield and yield component of sugar beet and barley under field conditions in response to fertilizer applications and seed inoculations of single, dual and multiple with N_2-fixing *Bacillus* (OSU-140 and OSU-142) and P-solubilizing *Bacillus* (M-13) bacteria* (average of 2001 and 2002)

Treatments		Sugar beet					Barley	
		Leaf Yield (t/ha)	Root Yield (t/ha)	Sugar Content (%)	White Sugar Content (%)	White Sugar Yield (t/ha)	Seed Yield (t/ha)	Total Biomass Yield (t/ha)
Control		23.28 f	44.37 f	19.08 a	16.76 a	7.43 e	2.70 f	7.13 e
NP		31.34 a	55.08 a	18.56 c	16.16 c	8.90 a	3.38 a	8.98 a
N		31.30 a	52.14 b	18.10 d	15.55 d	8.10 bc	3.08 b	8.58 b
OSU-140 (Nb_1)		27.06 cd	48.72 d	18.78 b	16.37 bc	7.97 cd	2.92 cd	7.84 cd
OSU-142 (Nb_2)		27.04 cd	49.24 cd	18.67 bc	16.30 bc	8.02 bc	2.85 de	7.93 cd
M-13 (Pb_1)		25.07 e	47.71 e	18.73 bc	16.45 b	7.84 d	2.83 e	7.75 d
$Nb_1 + Nb_2$		27.31 bc	49.87 c	18.68 bc	16.35 b	8.15 b	2.95 c	7.99 c
$Nb_1 + Pb_1$		26.37 d	49.26 cd	18.75 b	16.44 b	8.09 bc	2.94 cd	7.83 cd
$Nb_2 + Pb_1$		27.08 cd	49.65 c	18.72 bc	16.40 bc	8.14 b	2.90 de	7.85 cd
$Nb_1 + Nb_2 + Pb_1$		27.84 b	49.99 c	18.64 bc	16.33 bc	8.16 b	2.95 c	7.97 c
Source	D.F.	ANOVA						
Year (Y)	1	**	***	***	**	*	***	***
Error a	8							
Treatment(T)	9	***	***	***	***	***	***	***
Y × T	9	NS	NS	NS	NS	*	NS	NS
Error b	72							
Total	99							

Means followed with the same letter within each column are not significant different (Duncan's Multiple Range Test= 0.05); *, **, *** significant at 0.05, 0.01 and 0.001 probability levels, respectively; NS: not significant

Results

Bacterial inoculations and NP application significantly affected all the parameters in both years in sugar beet and barley. In sugar beet as an average of both years the highest leaf yields were obtained in N and NP plots representing increases over control of 34.4% and 31.3% followed by mixture of all three strains (19.6%) and dual inoculations (OSU-140 + OSU-142) (17.3%). P-solubilizing *Bacillus* M-13 gave the lowest value after control (Table 2). Of the bacterial inoculations, triple combination of N_2-fixing (*Bacillus* OSU-140 and *Bacillus* OSU-142) and P-solubilizing (*Bacillus* M-13) inoculation produced the highest leaf yield while P-solubilizing bacterium gave the lowest yield. On the average of both years, inoculation with the mixture of three bacteria increased sugar beet leaf yield by 3.0 and 11.0%, respectively when compared with single application of N_2-fixing and P-solubilizing bacteria. Root yields were also higher in N and NP plots. However, bacterial inoculations also significantly increased root yield (Table 2). Two year's data suggest that seed inoculation of sugar beet with OSU-140, OSU-142, M-13, OSU-140 + OSU-142, OSU-140 + M-13, OSU-142 + M-13 and OSU-140 + OSU-142 + M-13 increased leaf yield by 16.2, 16.1, 7.7, 17.3, 13.3, 16.3 and 19.6% as compared to the control and root yield by 9.8, 11.0, 7.5, 12.4, 11.0, 11.9 and 12.7%, respectively. N and NP application increased root yield up to 17.5 and 24.1%. Sugar beet receiving bacterial inoculations showed significant increases in leaf and root yield compared with control.

Quality parameters were also affected by the applications. Control plots gave the highest sugar content (19.08%) and white sugar content (16.76%) while N application gave the lowest sugar content (15.55%), bacterial inoculations having values lower than control but higher than N applied plots (Table 2). In both years, NP application gave the highest white sugar yield, and control plots gave the lowest white sugar yield (Table 2). However, sugar content and white sugar content of sugar beet from all inocula-

tions did not vary significantly. In comparison with control plots, NP application gave the highest white sugar yield in both years whereas P-solubilizing bacteria alone produced the least increase. No statistical difference was recorded between N applied plots and the plots received dual inoculation with N_2-fixing *Bacillus* OSU-142 and P-solubilizing *Bacillus* M-13 in term of white sugar yield.

Among the bacterial inoculations, the best treatment was the combination of all three strains, followed by dual inoculations N_2-fixing OSU-142 and OSU-140, and with a mixture of N_2-fixing OSU-142 and P-solubilizing M-13 in terms of root and sugar yields. Dual inoculation with N_2-fixing OSU-140 and P-solubilizing M-13 increased root and sugar yield compared P-solubilizing M-13 single ones, but being no more effective than single inoculation of N_2-fixing strains. Also, mixed inoculation of N_2-fixing and P-solubilizing bacteria enhanced leaf, root and sugar yields of sugar beet, compared with single inoculations with *Bacillus* OSU-140 or *Bacillus* M-13.

Treatments significantly increased also barley biomass and seed yields (Table 2). NP application produced the highest seed and biomass yield and control plots gave the lowest yields. In barley NP application produced the highest seed and biomass yields followed by N application and combined inoculations with three bacteria. Grain and total biomass yields of barley inoculated with P-solubilizing *Bacillus* M-13 in combination with N_2-fixing *Bacillus* OSU-140 and OSU-142 were significantly higher than that of inoculated with *Bacillus* M-13 alone.

Discussion

Two years of trials indicated that single inoculations with N_2-fixing bacteria increased sugar beet root yields by 9.8–11.0% and barley seed yields by 5.6–8.1% over control. Inoculation with phosphate solubilizing bacteria alone increased yields only by 7.5% and 5.5% in sugar beet and barley respectively. Dual inoculation of N_2-fixing bacteria with P-solubilizing bacteria gave yield increases by 11.9–12.4% in sugar beet and 7.4–9.3% in barley. Mixed inoculation of two N_2-fixing bacteria in combination with P-solubilizing bacteria gave yield increases over control by up to 12.7% in sugar beet and 9.3% in barley. NP applications, however gave yield increases up to 20.7–25.9% in both crops.

Compared with single inoculation of phosphate solubilizing M-13, sugar beet leaf, root and white sugar yield significantly increased in the combinations with two or three bacteria. Dual inoculation of N_2-fixing *Bacillus* OSU-140 and OSU-142, and/or mixed inoculations with three bacteria significantly increased grain yield of barley compared with single applications of *Bacillus* OSU-142 and M-13.

In this study, inoculation with dual mixtures with N_2-fixing and P-solubilizing bacteria had no significant effect on barley grain and total biomass yields compared with single inoculations of N_2-fixing bacteria. In sugar beet, dual inoculation with N_2-fixing OSU-140 and P-solubilizing M-13 gave similar leaf, root and white sugar yields to single applications of N_2-fixing bacteria. Differences in terms of leaf yield were, however, not significant between single inoculation with N_2-fixing bacteria and dual inoculations with N_2-fixing and P-solubilizing bacteria. Some of the previous studies showed that mixture inoculations had no comparative advantage over single cultures in wheat (Han and New, 1998) and in other crops (Chiarini et al., 1998). In contrast to other studies suggesting that combined culture inoculants significantly increased grain and dry matter yields as compared with single inoculation of individual organisms in sorghum (Alagawadi and Gaur, 1992), and in sugar beet and barley (Çakmakçi et al., 1999). P-solubilizing bacteria in combinations with N_2-fixing organisms were expected to improve the P nutrition of plants and, therefore, stimulate plant growth (Whitelaw et al., 1997).

Dual inoculation with N_2-fixing (OSU-140) and P-solubilizing (M-13) bacteria produced similar results to single applications of N_2-fixing bacteria probably stemmed from inter-species competition and/or interaction. In dual inoculations, OSU-142 performed better than OSU-140 for sugar beet. The rhizosphere competence of native bacteria for C sources was major determinant for the success of inoculants (Gyaneshwar et al., 2002). As free living, non-photosynthetic bacteria depend on soil organic matter as a food source, enhanced bacterial populations in the mixtures possibly increased competition for energy sources in the soil. Plant growth promoting activity was partially independent of bacterial population size on roots (Chiarini et al., 1998). The nutrient competition between plant and high bacteria population probably limited plant growth (Oliveira et al., 2002). Mixed microbial cultures allow their components to interact with each other synergistically, thus, stimulating each other through physical or biochemical activities (Vassilev

et al., 2001). The interaction of N_2-fixing bacteria with other bacteria could also inhibit their diazotrophic activity (Rojas et al., 2001). Soil microbial cultures with similar or different functions might express beneficial actions in a soil or rhizosphere (Bashan, 1998).

Root yields were lower in the first year due to relatively dry conditions prevailed. Greater performance of the bacteria tested in our study under relatively wetter conditions prevailed in the second year may substantiate the suggestion that the nitrogen fixing activity of free-living bacteria may strongly dependent on favorable moisture and temperature conditions (Hubbel and Kidder, 1998).

In the plots receiving bacterial inoculations, sugar content and white sugar contents of sugar beet were higher compared to the plots receiving 120 kg ha^{-1} N fertilizer application. Excepting N_2-fixing OSU-140 single inoculation, N_2-fixing bacteria gave comparable sugar yields in all combinations to N applications. More balanced uptake of minerals in the presence of N_2 fixation rectified the quality as compared with reduced quality in the plots receiving N fertilizer application. Moreover, lower leaf/root ratio in bacterial inoculations in contrast with N applications could be of importance in indicating earliest harvest maturity dates in the areas with relatively shorter vegetation periods. Mineral N application enhances leaf growth but reduced sugar contents compared with bacterial inoculations. Correlations of top leaf mass as a percentage of the total plant mass were significant with sugar content (−) and impurity content of roots (+) of sugar beet harvested in successive period (Maslaris et al., 1997). There is concern within the sugar industry that too much nitrogen is currently being applied to the beet crop. This is because, excessive use has deleterious effects on the quality of the harvested beet that make the crop less profitable for both grower and processor. In particular, beet given too much fertilizers N contains smaller content of sugar and higher of α-amino nitrogen compounds, both of which decrease the efficiency of sugar extraction.

In contrast with other combinations, dual inoculation with N_2-fixing bacteria of OSU-140 and P-solubilizing M-13 did not always significantly increase leaf, root and sugar yield of sugar beet and grain and biomass yield of barley compared to single applications both N_2-fixing bacteria. Inoculation with N_2-fixing bacteria of OSU-140 and OSU-142, however, increased sugar beet and barley yields by up to 11.0% and 9.8% compared with uninoculated control receiving no fertilizers. Inconsistent advantages of N_2-fixing and P-solubilizing bacterial combinations in trials may be due to their antagonistic effects and/or the inefficiency of phosphate bacteria although laboratory trials clearly indicated that M-13 dissolved phosphate. Further work, however, is required regarding the potential of phosphate bacteria involving more strains. In view of need for environmental friendly nitrogen sources, N_2-fixing organisms are of importance for non-legume crops.

Greater attention should be paid to new combinations of N_2-fixing and P-solubilizing bacteria for improvement of biofertilizers efficiency. There is a need to develop specific plant–bacterial strain combinations with greater effectiveness under a wide range of experimental conditions. The mechanisms explaining the synergistic interaction should be further investigated to elucidate the biochemical basis of these interactions, warranting further studies including more bacteria with similar or different metabolic activity depending on the specific soil or rhizosphere systems.

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Section editor: *D.E. Crowley*