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Full Length Research Paper

Determination of optimum dose and frequency of application of free-living diazotrophs (FLD) on lettuce

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Free-living diazotrophic isolates L1 (*Bacillus cereus* Frankland) and Br2 (*Bacillus subtilis* Ehrenberg Cohn) were evaluated in greenhouse trials for their optimum dose and frequency of application on seedlings of a single lettuce variety (Great Lakes) in a composted pine bark medium. Bacterial isolates were grown in Burke's broth and were applied as a drench at four different doses (10^5, 10^6, 10^7 and 10^8 colony forming units (cfu) ml^{-1}) and at different frequencies of application (1, 7, 14 and 21 days). Two months later, wet weight, dry weight and plant N levels were measured. Lettuce growth and plant N level responded positively to both bacterial isolates inoculated at 10^6cfuml^{-1} weekly and fortnightly. Isolate Br2 inoculated at 10^6cfuml^{-1} fixed 32.4% plant N when applied weekly and 26.7% when applied every two weeks. Isolate L1, at the same dose, applied weekly or every two weeks, fixed 27.7 and 29.1% of plant N requirement, respectively. The lettuce seedlings responded less well to a higher dose at 10^8cfu ml^{-1} of either isolate applied weekly and every two weeks. The response to dosage depended on frequency of application. Doses of 10^6 cfu ml^{-1} of both isolates applied weekly or every two weeks had the best effect on lettuce seedling growth and plant N.

Key words: Free-living diazotrophs, lettuce, optimum dosage, frequency of application.

INTRODUCTION

Nitrogen is often a limiting nutrient for crop production. However, the cost of nitrogen fertilizers increased drastically in line with oil prices. Extending biological nitrogen fixing ability to non-legumes by using free-living diazotrophs would be a useful technology for increasing crop production (Kennedy and Tchan, 1992).

A number of microorganisms are known to have beneficial effects on plant growth. Plant growth promoting rhizobacteria (PGPR) are commonly used as inoculants for improving the growth and yield of agricultural crops (Khalid et al., 2004). Among these are plant growth promoting rhizobacteria that form a symbiotic relationship with plants such as nitrogen fixing *Rhizobium* sp., as well as free-living diazotrophs (FLD) associated with the roots of grasses (Mishustin, 1970; Kloepper et al., 1980; Glick, 1995).

In a greenhouse study on sugarbeet, three different *Bacillus* isolates fixed nitrogen and increased growth (Çakmakçi et al., 2006). Similarly, inoculation with a strain of *Bacillus* sp. also increased growth of roots and shoot parts of rice plants (Beneduzi et al., 2008). In another study, Hafeez et al. (2006) noted that selected *Bacillus* sp. used as bio-inoculants on wheat resulted in increases in plant biomass, root length, and plant nitrogen and phosphorous content.

Narula et al. (2005) found that inoculation with *Azotobacter* sp. increased wheat and cotton yield, dry weight, and plant nitrogen. Similarly, nitrogen concentration in wheat grain and root tissue may increase due to *Azotobacter* bioinoculants (Kader et al., 2002). In another study, inoculation of *Azotobacter chroococcum* Beijerinck onto *Brassica napus* cv. ISN-129 produced an increase in seed yield and total dry matter when no external nitrogen was applied (Prabhjeet and Bhargava, 1994). Other FLD detected in association with plant roots and found to fix nitrogen include: *Acetobacter diazotrophicus,* Gillis et al., *Herbaspirillum seropedica,* Baldani et al. (James and Olivares, 1998), *Azoarcus* sp. (Hurek et al., 2002) and *Azospirillum* sp. (Steenhoudt and Vanderleyden, 2000).

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Inoculation of *Azospirillum* to cereals and non-cereal species typically results in increases in plant dry weight and in the amount of nitrogen in shoots (Baldini and Döbereiner, 1980; Albrecht et al., 1981; Bashan, 1986; Kapulnik et al., 1987). Similarly, significant increases in growth and dry matter were obtained by Venkateswarlu and Rao (1983) for pearl millet following inoculation with *Azospirillum brasilense* Corrig. (Tarrand et al. 1979). Recent studies have shown that several other bacterial species such as *H. seropedicae*, and *Burkholderia* spp. increased fresh weight and plant nitrogen of rice through biological nitrogen fixation (Baldani et al., 1997; Baldani et al., 2002; Rodrigues et al., 2008).

The aim of this study was to evaluate the effectiveness of two FLD isolates on lettuce growth under greenhouse conditions and to evaluate the effects of different doses and frequencies of application, on lettuce biomass and plant nitrogen levels in the lettuce plants.

MATERIALS AND METHODS

Preparation of inoculants

From frozen stocks, FLD bacterial strains L1 and Br2, were grown in Burke's medium (Bergensen, 1980). Isolate L1 was previously identified as *B. cereus* and demonstrated good multi-crop effects on plant growth and nitrogen-fixation activity. Isolate Br2 (*Bacillus subtilis* (Ehrenberg) Cohn) was supplied by Dr Brendon Neumann[1]. The bacterial inocula were produced by growing the bacteria in 250 ml of Burke's broth, on a rotary shaker (150 rpm for 72 h at 28°C). In the log phase of growth, bacterial suspensions were centrifuged (10,000 × g for 10 min at 4°C) and washed three times in phosphate buffer. Final cell pellets were diluted with sterile distilled water and cell numbers were adjusted to 10^5, 10^6, 10^7 and 10^8 cfu ml^{-1} for each of the two bacterial isolates, as determined by dilution plating and use of a counting chamber. This procedure was repeated each time fresh cell suspensions were needed. Five milliliters of each bacterial suspension was applied to each seedling.

Microbial inoculation

The prepared cell suspensions (10^5, 10^6, 10^7 and 10^8 cfu ml^{-1}) of both FLD isolates were added separately to each of the eight conical labeled flasks. Two grams of a sticker, gum Arabic[2], were dissolved in 100 ml of tap water and allowed to stand for 1 h. This allowed the substance to dissolve and form a homogeneous suspension. The suspension was further divided into eight 50 ml beakers, each containing 10 ml aliquots of the sticker. To each of the beakers, 10 ml of the bacterial cultures were added separately, and stirred. This resulted in a volume of 20 ml of sticker-bacterial suspension in each of the eight beakers of a 1:1 suspension.

Seed coating took place in a plastic bag. The bag was filled with 200 g seeds. The bacterial-sticker suspension was added at a rate of 0.1 mlg^{-1} of seeds. The use of a sticker was to increase the amount of inoculants that would adhere to seeds in order to increase the number of bacteria stuck onto each seed. The bag was closed in such a way as to trap as much air as possible. The bag was shaken for two minutes until all the seed were uniformly wetted with the sticker suspension. The bag was opened and the seed spread onto paper towels and air-dried overnight. The coated seeds of lettuce (cultivar Great Lakes) were planted in 32 pots filled with composted pine bark. The pots were watered with tap water and soluble fertilizer was applied at a rate of 0.224 $g\ell^{-1}$ KH_2PO_4, 0.149 $g\ell^{-1}$ K_2SO_4, 0.324 $g\ell^{-1}$ KCl, 0.203 $g\ell^{-1}$ $MgSO_4$, 1 $g\ell^{-1}$ $CaCO_3.12H_2O$ to give 300 $mg\ell^{-1}$ and 50 $mg\ell^{-1}$ and left in a greenhouse (20 to 25°C) till harvest. The seedlings were subsequently drenched with the FLD isolates after seedling emergence at different doses and frequencies. Seedlings were dosed with 10^8, 10^7, 10^6, 10^5 $cfuml^{-1}$of bacteria at a rate of 1 ml liquid culture per plant. Doses were applied every 7, 14, and 21 days. The experiments used a complete randomized block design with three replicates for the three factors tested: Inoculation with the FLD isolates Strain L1 (*B. cereus*) or Strain Br2 (*B. subtilis*); doses of 10^5, 10^6, 10^7 and 10^8 $cfuml^{-1}$; and frequencies of 1, 7, 14 and 21 days: 1 = seed treated prior to plant, 7 - every week, 14 = every two weeks and 21 = every three weeks, after emergence of seedlings, respectively. The treatments were applied for one month in order to establish the bacteria. Two months later, wet and dry biomass weight and plant nitrogen were measured.

Controls

Two controls were set up in this study. Untreated seeds were planted in nine pots filled with composted pine bark and received water only. The other nine pots served as a positive control and were supplemented weekly with a balance NPK fertilizer called 3:1:3 (38) Complete[3].

Total nitrogen analysis

The analytical technique of Willis et al. (1996) was used.

Statistical analysis

The GenStat 9th edition was used for analysis of variance. When the F-test was significant, the treatment means were compared using the least significant difference (LSD) test.

RESULTS

The response of lettuce seedlings to treatments with isolate Br2 (*B. subtilis*) at a dose of 10^5 cfu ml^{-1} applied once as a seed treatment were not significant (p=<0.001) for growth compared to control-none. The single inoculation (seed treated) was not effective (Table 1). However, when isolate Br2 at doses of 10^6, 10^7, 10^8 cfu ml^{-1} was applied at all frequencies, there was significant increase in plant nitrogen (P<0.001).

Isolate L1 (*B. cereus*) at doses of 10^5, 10^6, 10^7 cfu ml^{-1} applied at all frequencies was significantly able to increase plant nitrogen of lettuce seedlings relative to the untreated and unfertilized control (P<0.001). Moreover, doses of 10^8 applied at all frequencies except at 21 days

[1] *Dr. Brendon Neumann, PHP (Pty) Ltd., Nottingham Rd., South Africa.*

[2] gum Arabic sticker, from *Acacia* sp., SIGMA

3 Ocean Agriculture (Pty) Ltd. P.O. Box 741, Muldersdrift, 1747, South Africa

Table 1. Response of lettuce (wet and dry weight and plant N) after two months in a greenhouse to varied dosages and frequencies of application of two FLD isolates.

FLD bacteria	Doses (cfu ml^{-1})	Frequency (weeks)	Wet weight (g)	Dry weight (g)	Plant N (mg g^{-1})
Br2 (*B. subtilis*)	10^5	1	2.4(0.50) ab	0.9(0.27) ab	2.0(0.46) a
		7	7.4(0.92) fghi	3.4(0.64) fghijk	6.1(0.84) defgh
		14	6.7(0.88) efghi	3.0(0.61) defghijk	4.8(0.76) cdefgh
		21	3.9(0.68) bcde	1.5(0.39) abcd	2.5(0.54) bc
	10^6	1	4.2(0.71) bcdef	1.4(0.39) abcd	3.4(0.63) bcde
		7	9.7(1.03) i	5.1(0.78) k	9.2(1.01) h
		14	8.2(0.96) ghi	3.9(0.69) hijk	7.5(0.92) fgh
		21	7.4(0.91) fghi	3.2(0.61) efghijk	6.5(0.85) defgh
	10^7	1	5.7(0.82) cdefghi	2.5(0.54) cdefghij	4.7(0.75) cdefgh
		7	7.6(0.92) fghi	3.8(0.67) hijk	6.3(0.86) efgh
		14	7.4(0.92) fghi	3.2(0.62) fghijk	6.0(0.84) defgh
		21	6.0(0.83) cdefghi	2.5(0.52) cdefghij	4.8(0.76) cdefgh
	10^8	1	7.4(0.91) fghi	3.6(0.64) fghijk	6.2(0.83) defgh
		7	4.1(0.67) bcde	2.1(0.44) abcdef	3.0(0.56) bc
		14	6.1(0.84) cdefghi	2.5(0.54) cdefghij	4.2(0.70) bcdefg
		21	6.2(0.83) cdefghi	2.8(0.54) cdefghij	5.3(0.74) cdefg
L1 (*B. cereus)*	10^5	1	5.54(0.79) cdefgh	2.34(0.49) abcefgh	5.18(0.74) cdefg
		7	6.49(0.86) defghi	2.8(0.57) defghijk	5.37(0.80) cdefgh
		14	5.26(0.77) bcdefgh	2.41(0.50) cdefghij	4.75(0.68) bcdef
		21	4.64(0.71) bcdef	1.97(0.45) abcefg	3.87(0.63) bcde
	10^6	1	6.68(0.86) defghi	3.07(0.50) defghijk	5.8(0.79) cdefgh
		7	8.53(0.97) ghi	4.38(0.72) jk	7.87(0.94) fgh
		14	8.69(0.98) hi	4.24(0.71) ijk	8.27(0.95) gh
		21	5.7(0.79) cdefgh	2.39(0.49) abcefghi	5.1(0.72) cdefg
	10^7	1	4.96(0.76) bcdefg	2.01(0.46) abcefg	4.35(0.73) cdefg
		7	6.17(0.84) cdefghi	2.68(0.54) cdefghij	5.32(0.76) cdefgh
		14	5.88(0.83) cdefghi	2.5(0.53) cdefghij	4.52(0.74) cdefg
		21	6.04(0.84) cdefghi	2.9(0.58) defghijk	5.57(0.79) cdefgh
	10^8	1	6.27(0.84) cdefghi	2.57(0.53) cdefghij	5.2(0.79) cdefgh
		7	3.7(0.66) bcd	1.49(0.39) abcde	2.73(0.54) bc
		14	3.25(0.63) ab	1.18(0.34) abc	2.93(0.59) bcd
		21	5.85(0.82) cdefghi	2.56(0.53) cdefghij	5.43(0.78) cdefgh
Control			1.69(0.42) a	0.71(0.23) a	0.86(0.27) a
NPK			35.48(1.56) j	18.39(1.29) l	28.37(1.47) i
P			<0.001	<0.001	<0.001
s.e.d			0.11	0.11	0.13
l.s.d.			0.22	0.22	0.26
C.V%			16.10	24.60	7.30

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Means with the same letter in the same experiment are not significantly different at P<0.05; Values in parenthesis represent transformed means using Log transformations.

did not significantly increase biomass when compared to the control-none (Table 1). Isolate L1 (*B. cereus*) at a dose of 10^8 cfu ml^{-1}, applied weekly did not significantly increase plant nitrogen relative to the control-none ($P<0.001$) (Table 1). Application of isolate L1 (*B. cereus*) at a dose of 10^5 cfu ml^{-1}, applied once at the time of planting and 21days later, supplied 18.3 and 13.6% of plant nitrogen requirement respectively. Moreover, 18.6, 27.7, 18.7 and 9.6% of plant nitrogen demand was provided by applying isolate L1 at doses of 10^5, 10^6, 10^7 and 10^8 cfu ml^{-1} at a frequency of once a week (Table 1)

When FLD isolate L1 (*B. cereus*) was applied to lettuce seedlings at 10^5 cfu ml-1 at all frequencies of application, 13.9 to 18.9% of plant nitrogen were generated by the bacteria. When Isolate L1 was applied at 10^6 cfu ml^{-1} weekly or every two weeks, it provided 27.7 to 29.1% of nitrogen demand. At doses of 10^7 and 10^8 cfu ml^{-1}, applied at 21 days, produced 19.1 and 19.9% of plant nitrogen, respectively. Less nitrogen was produced when Isolate L1 was applied at doses of 10^7 and 10^8cfu ml^{-l} weekly and every two weeks (9.6 and 10.3%; 15.0 and 18% of nitrogen demand, respectively) (Table 1). Significant difference was observed in frequency, concentration and the interaction of frequency × concentration applications ($P<0.001$).

DISCUSSION

At harvest after two months, there were significant differences in wet and dry weight of lettuce plants ($P<0.001$), as a result of inoculation with Isolates L1 and Br2 at various doses and frequencies.

At 10^5, 10^6, 10^7 $cfuml^{-1}$ weekly application of either isolates was the most successful frequency of application. Similar results were obtained by Okan and Labandera-Gonzalez (1994) who determined that the optimum concentration of an *Azospirillum* isolate was 10^7 cfu ml^{-1} for inoculation onto a range of different host plants. However, at 10^8 $cfuml^{-1}$, a single seed treatment or a seed treatment and a single drench at 21day was more effective than every 7 or 14 days application. According to Bai et al. (2002), co-inoculation of plant growth promoting rhizobacteria (PGPR) strains increased nodule number, plant dry weight and fixed nitrogen at optimal doses (10^8 cells per seedling). Okon and Itzigsohn (1995) also suggested that a bacterial concentration of 10^9 to 10^{10}cells g^{-1} or ml^{-1} was the optimum concentration of FLD bacteria when applied as a seed treatment, especially for crops with small seeds. The high dose and high frequencies were less effective than at low doses and low frequency. This is probably due to an increase in substrate requirements of the bacterial populations. It seems clear that there is an optimum dose × frequency for FLD.

Indeed, our study showed the application of FLD isolates at 10^5 $cfuml^{-1}$ doses at 1 day (seed treated) and 21 days application did not result in a significant increase in biomass in comparison to the untreated control. At a dose of 10^{-8} $cfuml^{-1}$ applied at a range of frequencies, there were significant increases in biomass, compared to the untreated control. These results provide evidence that the doses and frequency of application may affect the establishment of inoculum in the rhizosphere of plants. The most interesting result from a commercial perspective is that the dose of 10^8 $cfuml^{-1}$ applied by seed treatment was effective because this would be relatively cheap to manufacture, and as a treatment, it is easy and relatively cheap to apply, especially for field crops when drenching is not a realistic option, other than at planting.

Overall, the results demonstrated the beneficial effect of FLD isolates, increasing biomass and plant nitrogen content of lettuce compared to the control (un-fertilized and un-inoculated). However, biomass was still much less than the NPK treatment and it is unlikely that these FLD treatments will replace nitrogen fertilization in the immediate future in commercial agriculture.

In conclusion, this study showed that inoculation of lettuce with FLD Isolates L1 (*B. cereus*) and Br2 (*B. subtilis*) increased yield and nitrogen content in lettuce. Dose and frequency of application interacted significantly.

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