Short Communication

EFFECT OF GREEN MANURE AND BIOCONTROL AGENTS ON POTATO CROP IN CORDOBA, ARGENTINA

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SUMMARY

Field trials were conducted in commercial plots over two consecutive years in order to determine the effect of wild oat used as green manure and biocontrol agents, alone or combined, on potato yield, number of tubers and scab incidence. The biomass of green manure was added to the soil one and a half months before sowing. Potato seeds were dipped in liquid suspensions containing either *Bacillus subtilis* B-235 or *Trichoderma harzianum* Th-1, or a combination of both. Green manure was effective in promoting potato yield and reducing scab incidence. The combination of green manure and the liquid formulation of B-235 provided significantly improved results with a higher number of potato tubers and lower potato scab incidence. The combination of wild oat green manure and B-235 is thus a promising alternative to improve potato yield and reduce scab incidence.

Key words: Green manure, wild oat, potato crop, *Trichoderma harzianum*, *Bacillus subtilis.*

The province of Córdoba is one of the main potato-growing areas of Argentina (De la Casa *et al*., 1999) with a surface of *ca.* 20,000 ha given over to this crop and an average yield of approximately 22,000 kg ha^{-1}. Potato is traditionally grown in wide plains in the world and responds significantly to nitrogen (N) fertilization for tuber formation (Sincik *et al*., 2008). One of the major phytopathological hazards of potato production is the common scab caused by *Streptomyces* spp. To control this pathogen, chemicals are usually used in spite of their high cost and negative environmental impact. Current concerns related to the contamination of ground and surface waters by agrochemicals have emerged as a significant human health and environmental issue.

An alternative control method is green manure (GM), i.e. a crop used primarily as a soil amendment and a nutrient source for subsequent crops (Cherr *et al*., 2006). A legume GM may increase mineralization of N and induce higher tuber yield in the subsequent potato crop, although the full-season GM occupies a large proportion of the crop rotation and, thus, is an expensive production parameter (Sincik *et al*., 2008). Sakuma *et al.* (2004) selected the cereal wild oat (*Avena strigosa* cv. Hayoats) as GM before the potato crop, because of the high yield, the rich root systems and easy decomposition. The latter is particularly important in crops such as potato, which is very demanding in terms of soil suitability, fertilization and water supply. The use of autumn GM, such as cereals, is a way to improve the efficiency of space and resource utilization. Nevertheless, there is the risk that the net contribution of N will be too small to meet the needs of the following potato crop (Bath *et al*., 2006).

An important strategy to enhance yield is the introduction of beneficial microorganisms around the root system to increase resistance to pathogens, and improve growth and assimilation of nutrients, particularly nitrogen, phosphorus and potassium (Forchetti *et al*., 2010).

When plant-microbial populations are complementary, the host and its resident microorganisms appear to benefit from each other (Bakker and Schippers, 1987; Kloepper *et al*., 1980). Sturz (1995) and Sturz and Matheson (1996) have reported that rhizobacteria are able to exert a profound influence on the health and yield characteristics of potato crops, including tuber number and size, and disease resistance. *Trichoderma* spp. and *Bacillus* spp. are examples of microorganisms that exert beneficial effects on plant growth and development and elicit host defense against plant pathogens (Harman *et al*., 2004; Kloepper *et al*., 2004; Raaijmakers *et al*., 2009). In Argentina, strains of *T. harzianum* and *B. subtilis* used as biocontrol agents (BCAs) have been shown to reduce the impact of *Rhizoctonia* disease and to increase potato yield in the field (Gasoni *et al*., 2001).

The goal of this study was to determine the effects of wild oat as GM, alone or in combination with BCAs, on potato yield, tuber number and scab incidence in field trials as part of a screening program for alternatives to

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chemical fertilizers. To this aim, two field experiments were conducted in 2002 and 2003 in a commercial farm located close to Córdoba city (Villa Retiro, 31°25´S, 64°11´W, Argentina). The soil type was a silty loam (7.8% sand, 68.4% silt, 25.4% clay) Entic Haplustoll Manfredi Series (INTA, 1987) with 2.9% organic matter content and pH 5.9. This soil had a history of five years of potato production with no rotation. The experiments were arranged as completely randomized blocks with five treatments and four replications, comprising 20 plots in total, each 4.5 m long x 1.60 m wide with 0.8 m between the rows. Approximately 300 seeds/m^2 of wild oat (*A. strigosa* cv. Hayoats) used as GM were sown in May (autumn) and *ca.* 2.2 kg fresh weight/m^2 were incorporated into the soil three months later (winter) when the plants were approximately 25-30 cm high, each having six or seven bunches. The soil was allowed to rest for one month and a half before potato was sown.

Two microorganisms used as BCAs in previous field trials (Gasoni *et al.,* 2001) were tested in this work, both recovered in Buenos Aires province, i.e. *B. subtilis* (B-235), from alfalfa, and *T. harzianum* (Th-1) from sclerotia of *Sclerotium rolfsii* Sacc. colonizing pepper tissues. The cultures were kept at the IMyZA-INTA collection. B-235 was conserved in 80% glycerol:20% water at -80°C, and Th-1 in a soil medium (Butler, 1980) at -20°C.

Trichoderma harzianum was grown in flasks containing 4,000 ml of BASE S liquid broth: 1.0 g KH_2PO_4 (Cicarelli Laboratories, Argentina), 0.5 g $MgSO_4.7H_2O$ (Cicarelli), 0.5 g KCl (Cicarelli), 0.01 g $FeSO_4.7H_2O$ (Anedra, Argentina), 0.01 g $ZnSO_4.7H_2O$ (Anedra), 0.005 g $CuSO_4.5H_2O$ (Anedra), 20.0 g sucrose (Cicarelli), 4.0 g yeast extract (Oxoid, UK)), 1000 ml distilled water (pH adjusted at 6.2 before autoclaving) prepared according to Cozzi and Gasoni (1997). *B. subtilis* B-235 was grown in 4,000 ml of BM liquid broth: 2.5 g NaCl, 1.0 g KH_2PO_4, 2.5 g K_2HPO_4, 0.25 g $MgSO_4.7H_2O$, 0.1 g $MnSO_4.H_2O$, 5.0 g glucose (all from Ciccarelli Laboratories, Argentina) 4.0 g yeast extract (Oxoid, UK), 1,000 ml distilled water (pH adjusted to 7.0 before autoclaving) prepared according to Gasoni *et al.* (1998). Liquid cultures were developed on a floor orbital shaker (350 rpm) at 28ºC. After three days of agitation, each inoculum containing about 10^8 propagules/ml or 10^8 CFU/ml were transported to the field site in refrigerated containers (6-10ºC).

Because of limited space and based on the results obtained in the experiments conducted in the region using the same BCAs (Gasoni *et al*., 2001), the treatments were: (i) potato seeds without any treatment; (ii) wild oat as GM and potato seeds without any treatment; (iii) wild oat and potato seeds inoculated with Th-1; (iv) wild oat and potato seeds inoculated with B-235; (v) wild oat and potato seeds inoculated with Th-1 and B-235.

Fifteen potato seeds of cv. Spunta were dipped for 30 sec in the liquid cultures containing the BCAs and sown 30 cm apart in each row during the second week of September (spring). The rows in the treatment plots were irrigated immediately after sowing with 3,000 ml of BCA suspensions (1,500 ml liquid broth: 1,500 ml water per row), then covered with soil. Harvest was in mid December (summer).

In both years, potatoes were grown using standard production practices except for the fact that the weeding was by hand and no fungicides were applied. Chemical fertilizers applied were 200 kg ha^{-1} of $(NH_4)_2HPO_4$ and 150 kg ha^{-1} of urea. The experimental field was irrigated when needed.

At harvest, tuber yield (kg $plot^{-1}$ and kg ha^{-1}), tuber number per plant, mean tuber weight (g), and scab incidence and severity (%) were recorded in each plot. The percentage of surface area covered by scab was estimated using the pictorial key of James (1971) for each tuber. Then, scab incidence was expressed as the percentage of tubers showing scab symptoms. Disease severity (%) was calculated as follows:

$$\frac{100 \times \Sigma\ [(\text{disease index}) \times (\text{number of diseased tubers})]}{(\text{Max. disease index}) \times (\text{n}^{\text{o}} \text{ of tubers examined})}$$

The experiment conducted in 2002 was repeated in 2003. The analysis of variance for tuber yield, tuber number per plant, mean tuber weight and disease severity was performed using the SAS general linear model (GLM) procedure (SAS Institute, USA). Data of both experiments were either analyzed separately for each year or pooled to have a larger number of repetitions and to verify the significance of treatment effects on potato crop. Means were compared using Tukey's multiple range test ($P \leq 0.05$). Disease severity data were transformed (arcsine applied to the square root of the proportion) prior to the statistical analysis. Actual values are shown. Potato scab incidence was analyzed using the Glimmix procedure (SAS) in order to handle the binomial responses. Means were compared using Tukey's multiple range test ($P \leq 0.05$).

Results from individual analysis showed that statistical differences between some treatments varied in 2002 and 2003. However, the mean response variable was similar in both years (Fig. 1 and 2), except for potato scab severity, which was higher in 2002 than in 2003 (Fig. 2), although the treatment effects were similar.

Results from combined analysis showed that potato yield was significantly increased (71%) when wild oat was incorporated into the soil, compared with the untreated plot, and that addition of BCAs to wild oat did not further increase the yield (Table 1). The treatment with wild oat alone showed no significant increase in tuber number, whereas the treatment using a combination of GM and B-235 led to the significant increase of 41%.

Treatments including the combination of GM and Th-1, or both microorganisms together, did not show significant differences compared to the GM treatment alone. Mean tuber weight was significantly increased by 51.2% when GM was incorporated into the soil, compared to the untreated plot. In the treatments combining GM and BCAs, the mean tuber weight showed lower values than in the GM treatment, although they were higher than in the untreated plot (Table 1).

Scab incidence was significantly decreased by 55.5% by GM treatment and by 74.7% with GM treatment plus B-235. The combination of GM and Th-1, or both microorganisms together, did not differ significantly from GM alone. Scab severity was significantly reduced by 58.1% by GM, whereas there were no significant effects when GM was combined with BCAs (Table 2). There was no interaction between treatments and years for any response variable.

The combination of methods to increase crop yield and improve pest control, especially when combining non-chemical methods, is the heart of integrated pest management (Katan, 2000). In our studies, the incorporation of *A. strigosa* cv. Hayoats as green manure was effective in promoting potato yield. It is important to point out that GM induced an increase in mean tuber weight but not in tuber number. Our results are in agreement with those obtained by Sakuma *et al*. (2004). Several authors have reported that green manures increase the mineralization of N and stimulated specific beneficial bacterial populations in the root zone, which also led to improvements in soil fertility, crop vigor, and higher tuber yield in potato crop (Båth *et al*., 2006; Cherr *et al*., 2006; Manter *et al*., 2007; Sincik *et al*., 2008).

In our experiments, the combination of GM and B-235 significantly increased the number of potato tubers. There are many factors that affect tuber formation. In our case, the increase in tuber number, when wild oat was combined with B-235, might be attributed to the ability of many *Bacillus* strains to produce plant hormones such as indole acetic acid, gibberellins, cytokinins and compounds that mimic the action of jasmonates (Forchetti *et al*., 2007). Abdala *et al*. (2000) reported that the levels of gibberelin and jasmonic acid-related compounds affected tuber formation. Gibberelins are involved in the initiation of the stolon, the maintenance of its diageotropic growth and the delay of tuberization. In the process of tuberization, a decrease in gibberellin level is a prerequisite for tuber formation. The mechanisms involved in the action of the combined treatment of GM and B-235 are not fully understood. The phenomenon might be related to the efficiency of GM to increase the uptake of macro and micronutrients and to the ability of B-235 to produce endogenous hormones, induced systemic resistance (ISR) modulators, and elicit plant defenses.

According to Pelacho and Mingo-Castel (1991) and Cenzano *et al.* (2005), ISR jasmonic acid-related compounds act as chemical signals that trigger senescence-related processes like tuberization, which take place after a sufficient vegetative development of the potato plant. Investigations on the signal transduction pathway

Table 1. Effect of wild oat as green manure, alone or combined with *Bacillus subtilis* (B-235) and *Trichoderma harzianum* (Th-1), on potato tuber yield, tuber number per plant and mean tuber weight. Potato seeds were inoculated by dipping for 30 sec in a suspension containing biocontrol agents. Within each row, means followed by the same letter are not significantly different according to Tukey´s multiple range test ($P \leq 0.05$).

Parameter	Year	Untreated	GM	GM + Th-1	GM + B-235	GM + Th-1 + B-235
Tuber yield (kg plot^{-1})						
	2002	14.250 b	23.560 ab	25.820 a	28.550 a	22.570 ab
	2003	12.200 b	21.650 a	23.330 a	22.900 a	21.150 a
	Pooled data*	13.225 b	22.605 a	24.575 a	25.725 a	21.860 a
Tuber yield (kg ha^{-1})	2002	19,792 b	32,722 ab	35,861 a	39,653 a	31,347 ab
	2003	16,944 b	30,069 a	32,403 a	31,806 a	29,375 a
	Mean	18,368 b	31,396 a	34,132 a	35,729 a	30,361 a
Number of tuber plant^{-1}	2002	4.471 b	5.222 b	6.339 ab	7.440 a	5.889 ab
	2003	4.271 b	4.721 ab	5.739 ab	6.539 a	5.538 ab
	Pooled data*	4.371 c	4.971 bc	6.039 ab	6.990 a	5.714 ab
Mean tuber weight (g)	2002	105.880 b	150.22 a	138.910 ab	129.540 ab	128.770 ab
	2003	95.660 c	154.478 a	140.275 ab	118.075 bc	127.853 ab
	Pooled data*	100.771 c	152.349 a	139.594 ab	123.807 b	128.311 b

Data from 2002 and 2003 experiments were pooled. GM = green manure (*Avena strigosa* cv Hayoats); Th-1 = *Trichoderma harzianum*-1; B-235 = *Bacillus subtilis*-235.

Table 2. Effect of wild oat as green manure, alone or combined with *Bacillus subtilis* (B-235) and *Trichoderma harzianum* (Th-1), on the incidence and severity of potato scab. Potato seeds were inoculated by dipping for 30 sec in a suspension containing biocontrol agents. Within each row, means followed by the same letter are not significantly different according to Tukey´s multiple range test (P≤ 0.05).

Parameter	Year	Untreated	GM	GM+Th-1	GM+B-235	GM+Th-1+B-235
Scab incidence (%)						
	2002	29.108 a	11.063 b	13.050 b	6.700 b	10.598 b
	2003	24.040 a	12.610 b	10.595 bc	6.768 c	10.275 bc
	Pooled data*	26.574 a	11.836 b	11.823 b	6.734 c	10.436 bc
Scab severity (%)	2002	0.665 a	0.257 ab	0.255 ab	0.067 b	0.107 b
	2003	0.197 a	0.102 ab	0.117 ab	0.057 b	0.072 b
	Pooled data*	0.431 a	0.180 b	0.190 b	0.062 b	0.090 b

* Data from 2002 and 2003 experiments were pooled. GM = green manure (*Avena strigosa* cv. Hayoats); Th-1 = *Trichoderma harzianum*-1; B-235 = *Bacillus subtilis*-235.

of elicited plants suggest that the ISR elicited by several strains of *Bacillus* is dependent on jasmonic acid (Kloepper, 2004).

In our study, scab incidence was reduced by treatments with GM alone and those combining GM and B-235. The reduction of scab severity with the incorporation of wild oat was also observed by Sakuma (2004). The differences in potato scab severity in 2002 and 2003 might be explained by the small differences in soil moisture during tuber development. Another explanation could be the presence of a different antagonist community in both field trials. The results of scab incidence and severity might be related to the use of GM as an energy source by beneficial microorganisms (Wiggins and Kinkel, 2005). These beneficial microorganisms may compete with the pathogen for resources or produce secondary antimicrobial metabolites or lytic enzymes that neutralize the pathogen attack, or may induce resistance in plants (Raaijmakers *et al*., 2009).

Other GMs have been reported to control *R. solani* and other pathogens of potato such as *Streptomyces scabies* and *Spongospora subterranean* (Larkin and Griffin, 2007). Cook and Baker (1983) and Wiggins and Kinkel (2005) found that GMs prevented an increase in scab disease in a potato monoculture when applied to naturally infected soils with low disease pressure. These authors showed that GMs induce the production of antibiotics by antagonistic microorganisms. Wiggins and Kinkel (2005) reported that the streptomycete community in GM-treated soils had a greater proportion of antagonistic streptomycetes with inhibitory activity against *S. scabies*, concluding that GMs increase the beneficial microflora and might selectively enrich the number of antibiotic producers or their activity. Finally, Weinhold and Bowman (1968) found that the use of soybean as green manure before potato crop favoured antibiotic production by *B. subtilis*, which inhibited the multiplication of *S. scabies*.

In previous studies we have observed that purified extracts from B-235 cultures, inhibited *R. solani* (Kobayashi *et al*., 1996), a pathogen detrimental to potato in many countries (Banville *et al*., 1996), and that B-235 was able to reduce radish and lettuce damping-off caused by this fungus in greenhouse and field trials (Gasoni *et al*., 1998, 2001). Therefore, the combination of GM and B-235 is also be beneficial in *Rhizoctonia*-infested soils. The effectiveness of this combination in controlling potato scab might be attributed to the production of antibiotics by the antagonistic strain B-235 or to the inability of the pathogen to compete for available nutrients. It is possible, however, that other mechanisms, such as induced resistance, may be involved in the combined treatments.

Whatever the mechanism, the present results convincingly illustrate the potential of the combined application of biocontrol agents with green manure to enhance yield and number of potato tubers and to reduce scab incidence.

ACKNOWLEDGEMENTS

We express our deep thanks to Professor J. Katan for helpful discussion, critical reading and correction of the manuscript. We wish to thank the Florensa Company for allowing the use of their field plots and we also acknowledge Lorena La Fuente and Estela Favret for the assistance in literature searches and preparation of the tables. This research was supported by the Japan International Cooperation Agency (JICA) and by the Instituto Nacional de Tecnología Agropecuaria (INTA).

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Received February 10, 2011
Accepted August 1st, 2011

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