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ORIGINAL ARTICLE

BACTERIAL BIOCONTROL OF *PHLEBANCHE RAMOSA* (L.) POMEL GERMINATION

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ABSTRACT: Series of laboratory experiments were conducted to study the efficacy of bacterial isolates and strains on Phelipanche ramosa seeds germination. All experiments were conducted at the Bio-pesticides and Bio-fertilizers Department, Environment, Natural Resources and Desertification Research Institute (ENDRI), National Centre for Research (NCR), Khartoum, Sudan. In all experiments treatments were arranged in a Randomized Complete Design (RCD) with four replicates. Twenty three soil borne bacterial isolates (15 organic nitrogen users and 8 mineral nitrogen users) and 3 bacterial strains (Bacillus circulans, B. megatherium var. phosphaticum and Azospirillum brasiliense) were tested. The results revealed that most of the organic nitrogen using bacterial isolates showed high biocontrol against P. ramosa germination compared to the mineral nitrogen using bacterial isolates. All bacterial isolates and strains inhibited P. ramosa germination. Among bacterial isolates and strains, isolate. ISO22M completely inhibited P. ramosa seeds germination (100%) in response to GR24 at the lower concentration as compared to the corresponding control. However, isolate ISO11S and Azospillirum brasilense strain enhanced germination by 18 and 26%, respectively in response to germination stimulant compared to the medium control. A promising strategy to control broomrape is the use of biological control methods via soil borne microorganisms.

INTRODUCTION

Broomrapes (*Orobanche* spp.) are achlorophyllous root-parasite flowering plants; therefore, they are completely dependent on their host for nutrition and water from the roots and causes serious yield losses (Joel *et al.*, 2007). *Orobanche* seeds require conditioning by exposure to moisture at temperatures between 15-20 C for at least 18 days for maximum germination (Zehar *et al.*, 2002). However, prolonged storage in these conditions causes the entry of the seeds to secondary dormancy (Van Hezewijk *et al.*, 1994). Apparently, during the conditioning period, seeds are released from dormancy through an increase in seed coat permeability and/or changes in the levels of endogenous germination promoters or inhibitors (Press and Graves, 1995; Zehar *et al.*, 2002).

A promising strategy to control broomrape is the use of biological control methods via soil borne microorganisms. Biological control is considered attractive for suppressing root parasitic weeds in annual crops, as the use of chemicals may cause injury to the host plant (Linke *et al.*, 1992). Rhizobacteria from faba bean and *Orobanche* spp were evaluated for their potentials as biocontrol agents for parasitic weeds. Among five bacterial isolates selected for pot trials, strain Bf 7- 9 of *Pseudomonas fluorescens* showed high biocontrol against *O. foetida* and *O. crenata* and positively influenced faba bean growth (Zermane *et al.*, 2007). Similarly, some *Rhizobium leguminosarum* strains have been reported to induce defense against *O. crenata* in pea through activation of the oxidative process, and production of possible toxic compounds, including phenolics (Muller-Stover and Kroschel, 2005). The inhibitory effects of the bacterial strains applied on faba bean could be attributed to a direct effect of the bacteria on the seed or indirectly through production of chemical (s) that is (are) toxic to the seeds. *Orobanche* infestation was influenced by the bacteria, AM fungi and the time when the observation was made. Faba bean inoculated with the combination of bacterial strains (B2) {TAL 1399 plus *A. brasilense}*, B3 (TAL 1399 plus BMP {*Bacllius megathirium var phosphaticum*}) alone or in combination with mycorrhiza fungi (AM) were completely inhibited *Orobanche* plant emergence (Hassan and Abakeer, 2013).The objective of this study was to study the efficacy of bacterial isolates and strains on *P. ramosa* seeds germination.

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MATERIALS AND METHODS

Series of laboratory experiments were conducted to study the efficacy of bacterial isolates and strains on germination stage of *P. ramosa*. All laboratory experiments were conducted at the Bio-pesticides and Bio-fertilizers Department, Environment, Natural Resources and Desertification Research Institute (ENDRI), National Centre for Research (NCR), Khartoum, Sudan.

Isolation of bacteria was performed on two different media which were prepared by dissolving A) Meat Peptone Agar (MPA): 5g Meat extract, 7.5g Peptones, 5g NaCl and 20g Agar were dissolved on liter of distilled water. MPA medium is usually used for isolation of organic nitrogen using bacteria. B) Starch Ammonium Agar (SAA): 10g Starch, 2g Ammonium sulphate, 1g Dipotassium hydrogen phosphate, 1g $MgSO_4$-$7H_2O$, 3g NaCl and 20g Agar were dissolved on liter of distilled water. SAA medium is usually used for isolation of inorganic nitrogen using bacteria. These media were prepared by autoclaving at 121°C under a pressure of 15 lb/in^2 for 15 minutes and were then cooled and poured in sterilized Petri dishes and kept for 24 hours before use.

In addition, non symbiotic Nitrogen fixer *Azospirillum brasiliense*, Phosphorus solubilizing bacteria *Bacillus megatherium* var. *phosphaticum* and Potassium solubilizing – Silicate bacteria *Bacillus circulans* strains were obtained from Bio-pesticides and Bio-fertilizers Department, ENDRI, NCR, Khartoum, Sudan.

The strigolactone analogue GR24 was provided by professor Zwanenberg, University of Nimijhen, the Netherlands. A stock (10 ppm) of GR24 was prepared by dissolving 1 mg in 1 ml acetone and completed to volume (100 ml) with sterile distilled water. The solution was kept refrigerated at 4ºC for further use.

Soil samples were collected from infested (Touti - Khartoum State) and non infested (Wad Rawa - Gaziera State) tomato fields. Fifteen soil samples were collected as follows: Twenty gram samples were randomly taken at 10cm depth from each site. Each large sample particles were crushed to uniform reasonable size and mixed thoroughly to make composite sample. The composite soil samples were placed each alone in polyethylene bags, labeled and transferred immediately to the laboratory. Soil samples were then air dried at room temperature. One millimeter mesh was then used to sieve the soil. Ten gram of each soil was dissolved in 90 ml of sterilized distilled water in conical flask. The contents were shaken well And the serial dilution was done.. The third to nine dilutions (10^{-3} to 10^{-9}) were prepared. Inoculums from dilution 10^{-7}, 10^{-8} and 10^{-9} were transferred to the agar surface of MPA and SAA plates, respectively. In each plate, the inoculums were spread over the agar surface using sterilized glass spreaders and the plates were incubated at 28°C. The periods of incubation were two days for MPA plates and 10 days for SAA plates.

Phelipanche ramosa seed was taken from JICA laboratory, Sudan University of Science and Technology. *P. ramosa* seeds were cleaned by placement in a measuring cylinder (1000ml) containing tap water with a few drops of liquid soap. Floating materials containing derbies and immature light seeds were discarded, the seeds were washed several times with tap water to free them from sand, then seeds were surface disinfected by soaking in 70% ethanol for 3 minutes, with continuous agitation and rinsed three times in distilled water subsequently, the seeds were immersed in 1% sodium hypochlorite for 2 minutes and rinsed three times in sterilized distilled water. The seeds were plotted dry on Whatman filter paper (No. I) under a Laminar flow hood, then were kept in sterilized glass vial at 10ºC for further studies. Glass fiber filter paper (GF/C) discs (8 mm diameter) were cut, wetted thoroughly with water and placed in an oven at 100ºC for I h. to be dried, the discs were placed in 9cm diameter Petri dishes lined with glass fiber filter paper (GF/C) and moistened with 5ml distilled water, or diluted media inoculated or non-inoculated with respective bacterial isolates or strains. About 20-20 surface disinfected *P. romosa* seeds were sprinkled on each of the glass fiber discs in each Petri dish. Then dishes were sealed with parafilm and placed in black polythene bags and incubated at 18ºC for 11 days. The discs containing *P. ramosa* seeds were blotted dry on normal filter paper (No. 1) to remove excess water and then transferred to sterile Petri dishes. Each disc was treated with 30 µl of GR24 at 5 and 10 ppm. Then seeds were re-incubated and examined for germination at 7 days later. Germination percentages were then calculated.

All experiments treatments were arranged in a Randomized Complete Design (RCD) with four replicates. Data on percentages germination was calculated for each disc and transformed to arcsine (Gomez and Gomez, 1984) and subjected to analysis of variance (ANOVA). Means were compared using the least significant difference (LSD) at 5% level.

RESULTS AND DISCUSSION

Effects of organic nitrogen using bacterial isolates on P. ramosa germination

In table (1), GR24 applied (5 and 10 ppm) to seeds conditioned in water and broth medium induced 39.67 – 39.82% and 38.79 – 39.38%, germination respectively. Generally all bacterial isolates applied during conditioning significantly inhibited seeds germination compared to control. ISO22M completely inhibited *P. ramosa* seeds germination in response to GR24 at 5 ppm and also significantly reduced seeds germination by 88.3% as compared to control in response to GR24 at 10 ppm. ISO7M significantly reduced germination between 60 – 86 % as compared to the medium control in response to GR24 at both concentrations.

Phelipanche conditioned seeds in water displayed high germination percentage (Table 1). Most of the bacterial isolates inhibited germination in response to GR24 compared to both controls. Among bacterial isolates ISO21M was the most suppressive.

In the second batch, *P. ramosa* seeds conditioned in water and medium induced germination 40.52 – 39.82% and 38.6 – 40.5%, respectively in response to both concentrations of GR24 (Table 2). All bacterial isolates applied during conditioning significantly inhibited germination in response to GR24 compared to both controls. ISO6M significantly (P 0.5) reduced germination by 71% in response to GR24 at the higher concentration compared to the medium control.

GR24 (5 and 10 ppm) applied for seeds conditioned in water induced highest germination value (38.79 – 46.15 %) compared to the medium control which induced germination to 37.28 – 38.61 % (Table 2). ISO6M, ISO25M and ISO8M significantly inhibited germination in response to GR24 at both concentrations as compared to the medium control. However, ISO6M completely inhibited germination at lower concentration of GR24.

Effects of mineral nitrogen using bacterial isolates and some bacterial strains on P. ramosa germination

In table (3) *P. ramosa* conditioned in water induced germination to 37.46- 48.07% in response to GR24. However, broth medium enhanced germination by 18% in response to GR24 at (10 ppm) compared to water control (Table 3). However, at the lower concentration of GR24 (5 ppm) seeds applied during conditioning in the broth medium reduced germination by 22% compared to water control. ISO4S, ISO5S, ISO7S and ISO8S inhibited germination, albeit not significantly in response to GR24 at 10 ppm compared to the medium control.

GR24 applied to seed conditioned in water exhibited 40.47 – 42.13 % germination. Seed conditioned in the nutrient broth and similarly treated with GR24 displayed 37.72 - 38.60% (Table 3). All bacterial isolates reduced germination compared to controls. Moreover, ISO7S inhibited germination by 61 – 62% in response to GR24 compared to the medium control.

P. ramosa seeds conditioned in water or broth medium and treated with GR24 induced 39.23% and 42.99% germination, respectively (Table 4). All bacterial strains and ISO11S isolate applied during conditioning were significantly inhibited germination in response to GR24 at both concentrations compared to both controls.

GR24 applied to seeds conditioned in water induced from 46 to 38.5% germination. Nutrient broth reduced germination to between 4 and 14% compared to water control (Table 4). *Bacillus megatherium* var. *phosphaticum* strain reduced germination by 26 – 29 % in response to GR24 at 5 and 10 ppm respectively compared to the broth medium. Moreover, ISO11S isolate and *Azospillirum brasilense* strain enhanced germination by 18 and 26%, respectively in response to GR24 at both concentrations compared to the broth medium control.

Early growth stage, such as seed germination is key phase for the development of *P. ramosa*. Inhibition of this early phase by naturally occurring compounds produced by microorganisms could be a general strategic option for management of parasitic plants (Boari and Vurro, 2004). Inhibition of germination and radical growth of *O. aegyptiaca* is thought to be mediated by a small alcohol soluble peptide (51000 Da) produced by *Azospirillum brazilense*. Competitive inhibition to the germination receptor is probably responsible for the peptide effect (Nun *et al.*, 2005). Barghouthi and Salman (2010) reported that *Pseudomonas aeruginosa* QUBC1, *P. fluorescens* QUBC3, *Bacillus atrophaeus* QUBC16, and *B. subtilis* QUBC18 significantly inhibited radical elongation (P50.01) of both *O. aegyptiaca* and *O. cernua* relative to control radicals, whereas *Microbacterium hydrocarbonoxydans* QUBC11 and *Ochrobactrum anthropi* QUBC13 showed less inhibitory effects. Previous in vitro studies Barghouthi *et al.*, (2000), showed that some of the tested bacteria *Enterobacter* sp. QUBC20 and *Serratia marcescens* QUBC6 may enhance in vitro germination of

Orobanche seeds in the presence of the germination stimulant GR24. Zermane *et al.* (2007) have shown that *P. fluorescens* Bf7-9 can suppress the pre-emergence of *O. foetida* Poir and *O. crenata* when tested in pot experiments. Bouillant *et al.* (1997) found that *A. brasilense* inhibited *Striga* seed germination. Lipophilic compounds extracted from log and stationary growth culture media prevent the germination of *Striga* seeds, whereas high concentrations of *A. brasilense* (10^{10} CFU ml-1) failed to block seed germination in presence of GR24 (Miche *et al.*, 2000). Ahonsi *et al.*, (2002) reported that co-inoculation of legumes (selected for capacity to induce germination of *S. hermonthica* seeds) with ethylene-producing, nonpathogenic rhizosphere *Pseudomonas* sp. and *Bradyrhizobium japonicum* are worth developing as a biological control option for *S. hermonthica* in maize.
Further, host range tests of these isolates, including important crop plants, are needed to be carried out. Currently, studies are in progress to evaluate the ability of these organisms in the greenhouse and under field conditions.

Table 1: Effects of organic nitrogen using bacterial isolates on *P. ramosa* germination in response to GR24, irrespective to the conditioning status (batch 1)

Treatments		Germination (%)	
Bacterial isolates	GR24 conc.	During conditioning	After conditioning
Water	10	39.82* (43.00)**	43.28 (49.25)
	5	39.67 (40.75)	45.72 (51.25)
Media	10	39.38 (40.25)	43.99 (48.25)
	5	38.79 (39.25)	37.72 (37.50)
ISO1M***	10	21.33 (17.75)	36.10 (34.75)
	5	17.60 (7.25)	38.63 (39.00)
ISO5M	10	26.77 (21.00)	33.56 (30.75)
	5	26.24 (20.25)	35.06 (33.00)
ISO7M	10	15.73 (9.75)	39.17 (40.00)
	5	5.28 (3.25)	38.41 (38.75)
ISO17M	10	27.33 (22.27)	41.69 (44.25)
	5	23.07 (17.75)	33.07 (30.50)
ISO18M	10	34.12 (31.00)	38.45 (39.00)
	5	21.05 (16.75)	32.65 (29.25)
ISO19M	10	22.15 (15.00)	37.88 (37.75)
	5	20.23 (15.50)	30.91 (26.75)
ISO21M	10	10.56 (6.50)	31.08 (27.25)
	5	18.92 (11.25)	31.14 (26.75)
ISO22M	10	4.61 (2.50)	34.58 (32.50)
	5	0.00 (0.00)	39.10 (41.00)
LSD (P 0.5)		10.206	6.421

* Transformed data **Data between brackets = origin data *** Bacteria isolated on MPA

Table 2: Effects of organic nitrogen using bacterial isolates on *P. ramosa* germination in response to GR24, irrespective to the conditioning status (batch 2)

Treatments		Germination (%)	
Bacterial isolates	GR24 conc.	During conditioning	After conditioning
Water	10	39.82* (40.75)**	46.15 (42.75)
	5	40.52 (42.25)	38.79 (39.75)
Media	10	40.53 (42.25)	38.61 (39.00)
	5	38.60 (39.00)	37.28 (36.75)
ISO4M***	10	26.41 (25.50)	30.36 (27.00)
	5	27.08 (21.00)	23.02 (15.50)
ISO6M	10	11.62 (5.50)	16.21 (10.50)
	5	24.98 (18.00)	0.00 (0.00)
ISO8M	10	26.91 (20.75)	28.27 (22.75)
	5	19.57 (11.25)	20.90 (16.75)
ISO10M	10	29.76 (24.75)	30.42 (25.75)
	5	25.41 (18.75)	32.38 (28.75)
ISO13M	10	27.74 (22.00)	39.69 (41.75)
	5	28.96 (24.00)	24.62 (22.25)
ISO14M	10	29.90 (25.50)	29.71 (24.75)
	5	28.11 (23.25)	36.39 (35.50)
ISO25M	10	30.42 (25.75)	18.28 (13.00)
	5	29.75 (24.75)	25.22 (18.50)
LSD (P 0.5)		6.030	10.356

* Transformed data **Data between brackets = origin data *** Bacteria isolated on MPA

Table 3: Effects of mineral nitrogen using bacterial isolates and strains on *P. ramosa* germination in response to GR24, irrespective to the conditioning status (batch 1)

Treatments		Germination (%)	
Bacterial isolates	GR24 conc.	During conditioning	After conditioning
Water	10	37.46*(26.43)**	42.13 (44.25)
	5	48.07 (55.00)	40.47 (42.25)
Media	10	45.84 (51.25)	37.72 (37.50)
	5	37.78 (27.00)	38.60 (39.00)
ISO1S***	10	50.22 (59.00)	31.64 (27.75)
	5	40.19 (41.75)	20.30 (13.50)
ISO2S	10	47.75 (54.75)	20.10 (15.25)
	5	43.77 (48.00)	33.37 (30.75)
ISO3S	10	50.29 (59.00)	29.32 (30.00)
	5	49.23 (57.25)	33.49 (30.50)
ISO4S	10	42.66 (46.00)	29.99 (25.25)
	5	45.02 (50.00)	34.90 (33.75)
ISO5S	10	40.65 (42.50)	19.53 (5.00)
	5	39.50 (40.50)	29.49 (24.75)
ISO7S	10	37.55 (37.25)	14.32 (11.50)
	5	34.85 (32.75)	15.00 (12.50)
ISO8S	10	41.08 (43.25)	32.24 (28.50)
	5	32.58 (31.50)	21.28 (17.75)
LSD (P 0.5)		8.425	11.833

* Transformed data**Data between brackets = origin data *** Bacteria isolated on SAA

Table 4: Effects of mineral nitrogen using bacterial isolate and strains on *P. ramosa* germination in response to GR24, irrespective to the conditioning status (batch 2)

Treatments		Germination (%)	
Bacterial isolates and strains	GR24 conc.	During conditioning	After conditioning
Water	10	39.23* (44.75)**	46.15 (42.75)
	5	42.99 (46.50)	38.51 (39.75)
Media	10	42.99 (46.50)	39.81 (41.00)
	5	40.37 (18.50)	38.56 (39.00)
ISO11S	10	24.63 (21.50)	44.86 (49.75)
	5	18.19 (10.00)	45.70 (51.25)
B. megatherium var. *phosphaticum*	10	27.46 (21.50)	28.29 (23.25)
	5	28.95 (23.50)	28.70 (23.25)
A. brasilense	10	26.32 (20.00)	46.87 (53.25)
	5	31.42 (27.50)	48.71 (56.00)
B. circulans	10	29.88 (25.75)	35.76 (34.25)
	5	26.52 (20.25)	39.51 (40.75)
LSD (P 0.5)		6.361	8.129

* Transformed data**Data between brackets = origin data *** Bacteria isolated on SAA

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