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Effects of bacterial isolates and strains on *Phelipanche ramosa* (L.) Pomel haustorium initiation

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Abstract

Laboratory experiments were conducted to study the efficacy of bacterial isolates and strains on *Phelipanche ramosa* haustorium initiation at the Bio-pesticides and Bio-fertilizers Department, Environment, Natural Resources and Desertification Research Institute (ENDRI), NCR, Khartoum, Sudan. Fifteen soil borne bacteria isolates (9 organic nitrogen users and 6 mineral nitrogen users) and 3 bacterial strains (*Bacillus circulans*, *B. megatherium* var. *phosphaticum* and *Azospirillum brasiliense*) were tested. All tested bacterial isolates and strains caused a significant (P≤ 0.5) reduction in haustorium initiation as compared to control in response to DMBQ (2, 6 dimethoxybenzequinone). Organic nitrogen using bacterial isolates ISO5M and ISO22M reduced haustoria by 32 to 54% as compared to the corresponding control. While the mineral nitrogen using bacterial isolate ISO1S and *Bacillus circulans* strain reduced haustorium by 37 to 45% compared to medium control. The most efficient isolates were identified as *Serratia odorifera* (ISO22M) with probability 95% and *Rhizobium radiobacter* (ISO5M) with probability 99%.

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Introduction

Broomrapes (*Orobanche* spp.) are root parasitic plants devoid of chlorophyll that develop a haustorium serving as both an attachment organ to host roots and a bridge for water, mineral and organic nutrient uptake from host vascular tissues. Branched broomrape (*Orobanche ramosa* L.) is the most widespread broomrape in the world, causing severe damage to several crops in the Mediterranean region and southeast Europe particularly to tomato (*Solanum lycopersicon esculentum* Mill) (Cagan and Toth, 2003). The haustorium attaches the parasite to the host, penetrates the host while keeping its own tissues intact, develops a vascular continuity between the host and parasite and ultimately provides the conduit through which host and parasite materials flow (Parker and Riches, 1993). The formation of haustorial connection between the parasite and the host vascular tissue allows broomrapes to withdraw water and photosynthates from the host. The haustorium develops when intrusive cells of the parasite penetrate host tissues, eventually reaching the conductive system of the host.

The ability to monitor haustorium development in vitro provided an assay for identifying host factors that induce haustoria; these have been termed xenognosins or haustorium-inducing factors (Riopel and Timko, 1995). The only haustorium-inducing compound isolated from host roots is 2,4-dimethoxybenzoquinone (DMBQ) (Chang and Lynn 1986). Hydrogen peroxide generated in Striga radicles provides the rate limiting substrate for host peroxidases that catalyse the conversion of their own cell wall components into haustoria-inducing benzoquinones (Keyes *et al*., 2000). Haustorial hairs do not develop in *Orobanche* but rather the external cells of the haustorium develop short secretory papillae that provide the adhesion surface (Joel and Losner-Goshen, 1994).

Several control strategies are employed against broomrapes but none has enjoyed unequivocal success. The methods are either uneconomic or hard to achieve, or result in incomplete protection. 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). Several mycoherbicidal organisms have been isolated (Amsellem *et al*., 2001; Goussous *et al*., 2009). Among the several microorganisms reported the isolate of *Fusarium oxysporum* var. *orthoceras* which gave some control of *O. cernua* (Bedi and Donchev, 1991) and *O. cumana* on sunflowers (Thomas *et al.,* 1999). In addition, two very promising isolates of *F. arthrosporioides* and *F. oxysporum* were isolated and found to be pathogenic to *O. crenata* and *O. ramose* on most vegetable crops (Amsellem *et al*., 2001). Zermane *et al.* (2007) reported that *Pseudomonas fluorescens* showed high biocontrol against *O. foetida* and *O. crenata* and positively influenced faba bean growth. The objective of this study was to evaluate the efficacy of bacterial isolates and strains on *P. ramosa* haustorium initiation, characterization and identification of the most efficient bacterial isolates.

Materials and methods

Laboratory experiments were conducted to study the efficacy of bacterial isolates and strains on haustorium initiation 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 in one 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 in one liter of distilled water. SAA medium is usually used for isolation of inorganic nitrogen using bacteria

(Tepper *et al.*, 1993). These media were prepared by autoclaving at 121°C under a pressure of 15 lb/in² for 15 minutes and were then cooled and poured in sterilized Petri dishes and kept for 24 hours before use.

Bacterial strains

Non symbiotic nitrogen fixer *Azospirillum brasiliense*, Phosphorus solubilizing bacteria *Bacillus megatherium* var. *phosphaticum* (BMP) and Potassium solubilizing – Silicate bacteria *Bacillus circulans* strains were obtained from Bio-pesticides and Bio-fertilizers Department, ENDRI, NCR, Khartoum, Sudan.

GR24

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 4°C for further use.

DMBQ

2, 6 dimethoxybenzequinone was provided by Prof. Sugimoto, from Kobe University, Japan. A stock solution (100 ml) was prepared by dissolving 1.68gm in 1ml acetone and completed to volume of 100 ml with sterile distilled water. The solution was kept refrigerated at 4°C for further use.

Soil samples collection

They 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. Large 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.

P. ramosa seeds

Were taken from JICA laboratory, Sudan University of Science and Technology. They were cleaned by placement in a measuring cylinder (1000 ml) 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 10°C 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 100°C 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 isolate or strain. About 20-30 surface disinfected *P. romosa* seeds were sprinkled in each glass fiber disc in each Petri dish. Then dishes were sealed with parafilm and placed in black polythene bags and incubated at 18°C for 11 days.

P. ramosa seeds conditioning

Seeds were conditioned in the presence and absence of bacterial isolates and/or strains as described above, were dapped on filter papers (whatman No. 1) and transferred to sterile Petri dishes. The discs containing P. ramosa seeds were treated with 30µl of

GR24 solution (0.1 ppm). The Petri dishes were sealed with parafilm and placed in the dark at 180C for 7 days. The discs containing the germilings were blotted dry on normal filter paper (Whatman No.1) then placed and inverted top-down on similar discs without P. ramosa seeds. The pair of discs was treated with 40µl solution of 2, 6 dimethoxy benzoquinone (DMBQ). The germilings resulted from seeds conditioned in water or broth medium were used as controls.

Statistical analysis

All experiments treatments were arranged in a Randomized Complete Design (RCD) with four replicates. Data on haustorium percentages were 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.

Identification

The most effective isolates were characterized and identified using the VITEK2 compact system method.

Results and discussion

Effects of bacterial isolates and strains on germination of P. ramosa

Fifteen bacterial isolates and three strains were selected on basis of a previous study concerning their effects on *P. ramosa* seeds germination. ISO22M gave the highest germination reduction by 96.89% followed by ISO19M and ISO8M (Table 1). However, ISO3S induced germination by 63.58%.

Table 1. Reduction percentage of *P. ramosa* germination as influenced by bacterial isolates and strains.

Bacteria	Reduction %	Bacteria	Reduction %
ISO1M*	- 28.75	ISO1S**	+ 34.88***
ISO4M	- 42.90	ISO2S	+ 42.30
ISO5M	- 48.12	ISO3S	+ 63.58
ISO8M	- 61.02	ISO7S	- 3.01
ISO14M	- 40.01	ISO8S	+ 0.53
ISO18M	- 40.15	ISO11S	- 49.85
ISO22M	- 96.89	BMP	- 13.37
ISO19M	- 61.62	*A. brasilense*	- 4.17
ISO25M	- 37.80	*B. circulans*	- 17.58

* Bacteria isolated on MPA
** Bacteria isolated on SAA
*** + Enhancement.

Table 2. Effects of organic nitrogen using bacterial isolates on *P. ramosa* haustorial initiation in response to DMBQ batch 1.

Treatments	DMBQ conc.	Haustoria (%)
Water	20	42.17* (50.75)**
	10	42.70 (46.00)
Media	20	43.86 (48.00)
	10	39.90 (41.25)
ISO1M***	20	23.84 (16.75)
	10	28.55 (23.00)
ISO5M	20	20.07 (15.50)
	10	22.52 (19.50)
ISO14M	20	28.02 (22.25)
	10	24.56 (17.50)
ISO18M	20	30.35 (25.75)
	10	35.21 (33.25)
ISO22M	20	29.83 (24.25)
	10	21.11 (15.00)
LSD (P≤ 0.5)		11.103

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

Effects of bacteria isolated on MPA medium on P. ramosa haustoria

In the first batch, seven bacterial isolates were evaluated for their ability to inhibit haustorial initiation in response to DMBQ. *P. ramosa* seeds conditioned in water or in nutrient broth displayed 43 - 42% and 40 – 44% haustoria, respectively (Table 2). At the higher concentration of DMBQ, all bacterial isolates caused a significant (P≤ 0.5) reduction in haustorium initiation as compared to both controls. ISO5M reduced haustoria by 44 - 54% compared to the broth medium control.

Table 3. Effects of organic nitrogen using bacterial isolates on *P. ramosa* haustorial initiation in response to DMBQ batch 2.

Treatments	DMBQ conc.	Haustoria (%)
Water	20	42.13* (40.75)**
	10	39.07 (39.75)
Media	20	39.20 (40.00)
	10	40.02 (41.50)
ISO4M	20	22.41 (14.75)
	10	26.33 (20.00)
ISO8M	20	27.66 (22.50)
	10	21.10 (17.00)
ISO19M	20	30.79 (26.25)
	10	33.03 (30.25)
ISO25M	20	18.72 (13.50)
	10	22.11 (15.00)
LSD (P≤ 0.5)		8.382

* Transformed data

**Data between brackets = origin data

*** Bacteria isolated on MPA.

In the second batch, DMBQ (10 and 20 µm) applied to *P. ramosa* seeds conditioned in water and nutrient broth displayed 39 – 42% and 40 – 39% haustoria, respectively compared to both controls (Table 3). All Bacterial isolates reduced haustorium significantly (P≤ 0.5) except ISO19M at the lower concentration of the stimulant as compared to the control. ISO25M was the most inhibitory, it reduced haustorium by 45-51% compared to the medium control.

Effects of bacteria isolated on SAA medium and some bacterial strains on P. ramosa haustoria

Six bacterial isolates and three strains were evaluated for their ability to inhibit haustorial initiation in response to DMBQ. *P. ramosa* seeds conditioned in water or in the broth medium displayed 40 - 39% and 39 – 40% haustoria, respectively (Table 4). Irrespective to DMBQ concentration, all bacterial isolates and strains caused significant (P≤ 0.5) reduction in haustorium compared to both controls. Moreover, at the lower concentration of DMBQ, ISO1S and *Bacillus circulans* strain caused the highest reduction of haustorium (45%) compared to the broth medium control.

Bacteria may control weeds by interrupting signals required for germination, radical elongation, haustorium formation, rhizotropism or attachment. Identification of the factors that may modulate bacterial inhibition of radical elongation will allow selection of bacterial isolates with promise as weed bio-control agents (Barghouthi and Salman, 2010). Hassan and Abakeer (2013) reported that faba bean inoculated with the combination between bacterial strains TAL1399 plus *A. brasilense*, TAL1399 plus BMP (*Bacllius megatherium* var. *phosphaticum*) alone or in combination with mycorrhiza fungi (AM) were completely inhibited *Orobanche* plant emergence. 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). At least a considerable delay in *Orobanche* infestation could be displayed on inoculation of faba bean with bacteria and AM fungi. Delayed infestation by the parasite was reported to cause less damage than early infestations (Hassan *et al.*, 2009). The inhibitory effects of the bacterial strains applied on *P. ramosa* could be attributed to a direct effect of the bacteria on the early developmental stages or indirectly through production of chemicals.

Table 4. Effects of mineral nitrogen using bacterial isolates and strains on *P. ramosa* haustorial initiation in response to DMBQ batch 3.

Treatments	DMBQ conc.	Haustoria (%)
Water	20	39.23* (43.25)**
	10	40.36 (42.00)
Media	20	40.23 (41.75)
	10	38.89 (39.50)
ISO1S****	20	24.11 (17.00)
	10	21.42 (13.75)
ISO2S	20	29.66 (24.50)
	10	28.42 (23.00)
ISO3S	20	23.73 (16.25)
	10	30.44 (26.00)
ISO7S	20	24.84 (18.50)
	10	30.34 (26.50)
ISO8S	20	24.08 (16.75)
	10	23.35 (16.00)
ISO11S	20	26.05 (19.75)
	10	24.80 (18.00)
B. megatherium var. *phosphaticum*	20	25.84 (19.25)
	10	31.86 (28.25)
A. brasilense	20	25.33 (18.75)
	10	25.45 (18.75)
B.circulans	20	25.41 (19.00)
	10	21.45 (13.75)
LSD (P≤ 0.5)		5.848

* Transformed data

**Data between brackets = origin data

*** Bacteria isolated on SAA.

Table 5. Biochemical characterization of the most potential bacterial isolates.

Test	Bacterial isolate		Test	Bacterial isolate	
	ISO5M*	ISO22M		ISO5M	ISO22M
APPA	-	-	SAC	+	+
ADO	-	-	dTAG	+	-
PyrA	-	+	dTRE	+	+
IARL	+	+	CIT	-	+
dCEL	+	+	MNT	-	-
BGAL	-	+	5KG	-	-
H_2S	-	-	ILATk	+	+
BNAG	-	-	AGLU	-	+
AGLTp	-	-	SUCT	-	-
dGLU	+	+	NAGA	-	-
GGT	-	-	AGAL	-	+
OFF	-	-	PHOS	-	-
BGLU	+	+	GIyA	-	-
dMAL	-	+	ODC	-	-
dMAN	+	+	LDC	-	-
dMNE	+	+	IHISa	-	-
BXYL	+	+	CMT	-	-
BALap	-	-	BGUR	-	-
ProA	-	-	O129R	-	+
LIP	-	-	GGAA	-	-
PLE	-	+	IMLTa	-	-
TyrA	-	+	ELLM	-	+
URE	-	-	ILATa	-	-
dSOR	-	+			

*Bacteria isolated on MPA.

Biochemical characterization and identification of the most potential bacterial isolates

On basis of the haustorium results the most effective isolates (ISO5M and ISO22M) were identified. They reduced *P. ramosa* haustorium by 44 – 47% respectively, compared to medium control. ISO5M identified as *Rhizobium radiobacter* (P = 99%) and ISO22M identified as *Serratia odorifera* (P = 95%) (Table 5).

S. odorifera is classified under the family *Enterobacteriaceae* and the only medically important species is *S. marcescens*. *S. odorifera* known as opportunistic pathogen (Ateba and Setona, 2011). *Agrobacterium radiobacter* is a member of the family *Rhizobiaceae*, it have been re-classified in the genus *Rhizobium* based on comparative 16S rRNA gene analyses (Young *et al.*, 2001). *R. radiobacter* is not characterized as a true human pathogen. It is an opportunistic pathogen of minor clinical significance (Namdari *et al.*, 2003).

Adoption of an integrated approach encompassing bacteria inoculation may provide a novel, cheap and easy method to apply for *P. ramosa* control under subsistence low-input farming systems.

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