

Application of Rhizobacteria for Induction of Systemic Resistance in *Brassica Campestris* L. Against Alternaria Leaf Spot Disease Caused by *Alternaria Brassicae*

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ABSTRACT

Alternaria leaf spot disease is an important disorder causing serious yield loss of *Brassica* spp. The current study was designed to evaluate the role of rhizobacteria in management of Alternaria leaf spot disease in *Brassica campestris*. For this purpose *B. campestris* seedlings were treated with fifteen rhizobacterial strains under greenhouse and field conditions. The effect of rhizobacteria on quantity of defence related biochemicals such as PAL, PPO, PO and total phenols was examined. The *Bacillus megaterium* ZMR-6 and *Pseudomonas fluorescens* RB4 treated plants were least affected by the pathogen under greenhouse condition. Moreover, *B. megaterium* ZMR-6 and *P. fluorescens* RB4 exhibited significantly higher amount of defence related biochemicals. The growth promoting traits of bacteria *B. megaterium* ZMR-6 and *P. fluorescens* RB4 showed these were capable to exhibit phosphate solubilization, siderophore production and auxin synthesis. The *B. megaterium* ZMR-6 exhibited ACCD activity but *P. fluorescens* RB4 devoid this character. The application of peat moss based carrier material of *B. megaterium* ZMR-6 and *P. fluorescens* RB4 under field conditions showed promising results for disease management and agro-economic aspects of *B. campestris*. The current research revealed that *B. megaterium* ZMR-6 and *P. fluorescens* RB4 induce systemic resistance against Alternaria leaf spot and enhance yield in *B. campestris*.

INTRODUCTION

Mustard is an important oil seed crop which fulfills about nineteen percent oil requirement of the world ^[1]. Alternaria leaf spots caused by *Alternaria brassicae* is one of the most destructive disease of this crop which cause 47% yield loss ^[2]. *A. brassicae* infect foliage and pods of the *brassica* crop resulting in lesion formation on leaves along with shattering of pods ^[3].

The disease causing pathogens reduce yield of the edible agronomic and horticultural crops over 10% ^[4]. *A. brassicae* is a soil borne cosmopolitan fungal pathogen which causes brown spot or Alternaria leaf spot in different crops ^[5,6]. Most of the crop cultivars are susceptible to this pathogen due to which proper management becomes a tricky task ^[7]. On the other hand, fungicides cause environmental issues and become ineffective because fungus may develop acquired resistance against these chemicals. These facts necessitate application of long lasting, economical and eco-friendly techniques for disease management ^[8]. The research regarding use of root colonizing bacteria as bio-control agents has gained popularity in recent era. The non-pathogenic rhizobacteria may be used as a substitute for chemicals to improve plant growth and resistance against different pathogens ^[9]. The metabolic activities of this plant growth promoting rhizobacteria (PGPR) including synthesis of growth hormones, siderophore production and phosphate solubilization improve growth and vigor ^[10-13]. Similarly, the PGPR with ACCD activity have an additional advantage of reducing production of ethylene and reactive oxygen species in plants under stress ^[14]. Hence, PGPR

with ACCD activity help plants to ameliorate the detrimental effect of biotic and abiotic stress [15,16]. These PGPR improve metabolic activities resulting in increased root growth and subsequent nutrients uptake in associated plants [17]. Some rhizobacteria also produce antimicrobial substances such as lactic acid, exotoxins, bacteriocins, antibiotics and lyso-zymes [18]. Researchers have found that non-pathogenic entities may stimulate defense system of plants prior to invasion of disease causing pathogen [19,20]. PGPR also generate antagonistic chemicals which decline injurious effects of pathogens and improve plant resistance against disease causing agents [21]. This plant defense mechanism is known as induced systemic resistance (ISR) and is very effective disease managing strategy [22]. PGPR may induce systemic resistance in associated plants by triggering pathogenesis related (PR) genes. PR genes are involved in activation of defense related enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine-ammonia-lyase (PAL). The phenolic compounds which are synthesized by PPO also play a pivotal role in development of plant resistance.

The aim of present study was to screen rhizobacteria which may manage Alternaria leaf spot in pepper and to elucidate the disease managing strategy.

MATERIALS AND METHODS

Evaluation of Bio-Control Efficacy of Rhizobacteria

The rhizobacteria and virulent strain of *A. brassicae* used during current research were obtained from University of the Punjab, Lahore, Pakistan (Table 1). The rhizobacteria inoculum (10^4 cfu/mL) was obtained by taking OD of 0.1 at 600 nm. Pathogen inoculum was prepared by harvesting both micro- and macro-conidia from seven days old cultures grown on sterile PDA media at concentration of 1×10^3 conidia/ml, by haemocytometer.

The pre-sterilized *brassica* seeds were sown in sterilized loamy soil placed in plastic pots. The soil of 30 days old seedlings was inoculated with 50 ml bacterial inoculum by soil drench method. Next day seedlings were sprayed with pathogen inoculum with the help of hand spray. The distilled sterilized water was used for control treatments. The effect of disease rating scale and subsequent disease index and control effects were analyzed after 25 days of inoculation [23,24].

Table 1. Potential of Bacterial spp. to control Alternaria leaf spot disease in *Brassica campestris*; Values are mean \pm standard deviation (n=3). Different letters represent significant difference at ($P \leq 0.05$) according to ANOVA and DNMR.

Bacterial spp.	Disease Index (%)	Control Effect (%)
<i>Acinetobacter sp.</i> 334	22.45 \pm 1.58bc	13.53 \pm 1.53f
<i>Acinetobacter sp.</i> CS9	18.01 \pm 2.31c ^e	24.73 \pm 3.83e
<i>Aminobacter aminovorans</i> 374	23.17 \pm 3.46bc	10.46 \pm 2.93fg
<i>Bacillus fortis</i> 162	18.16 \pm 2.86d ^f	28.21 \pm 2.10de
<i>Bacillus megaterium</i> ZMR-6	15.82 \pm 2.26f	46.14 \pm 3.37bc
<i>Bacillus subtilis</i> 170	16.41 \pm 3.92ef	35.51 \pm 3.62bc
<i>Bacillus subtilis</i> 189	17.00 \pm 2.60ef	31.42 \pm 2.91cd
<i>Bacillus thuringiensis</i> 199	17.26 \pm 0.96ef	32.37 \pm 2.73cd
<i>Bordetella pertussis</i> 263	20.74 \pm 1.76bc	17.25 \pm 1.16e ^f
<i>Burkholderia capacia</i> 337	21.87 \pm 1.75b ^d	12.32 \pm 1.53f
<i>Burkholderia cepacia</i> CS8	18.34 \pm 3.67d ^f	29.15 \pm 2.44de
<i>Enterobacter sp.</i> CS2	22.41 \pm 2.26b-d	13.23 \pm 2.72f
<i>Microbacterium lacticum</i> 261	25.30 \pm 3.43b	03.71 \pm 0.57h
<i>Pseudomonas fluorescens</i> 083	25.34 \pm 1.08b	06.58 \pm 1.05gh
<i>Pseudomonas fluorescens</i> RB4	13.83 \pm 3.05g	49.36 \pm 4.64a
Sterilized distilled water	56.64 \pm 4.23a	-

The bio-control effect (%) was determined as under:

$$\text{Bio-control effect (\%)} = \frac{\text{Disease index of pathogen control} - \text{diseased index of bacterial control}}{\text{Disease index of pathogen control}} \times 100$$

Preparation of Plant Extract

The prewashed plant samples (2 g) were frozen by using liquid nitrogen and ground to powder form with the help of mortar and pestle. The ground samples were re-suspended in cold phosphate buffer (0.05 M) containing polyvinylpyrrolidone (0.5 g) at pH 7.0 (1:5 plant tissues to buffer ratio). After homogenizing this mixture by vortex mixture, the homogenate was centrifuged at $14,000 \times g$ at $4^\circ C$ for 20 min [25]. Subsequently the supernatant were used for evaluation of PO, PPO and PAL activity.

Analysis of PO Activity

PO activity was determined by measuring the guaiacol oxidation in the presence of hydrogen peroxide (H_2O_2) as described by Fecht-Christoffers et al. [26]. For evaluation of PO activity, 100 μ l crude plant extract was mixed with 3 ml solution containing 1 ml each of 0.25% guaiacol H_2O_2 (0.1 M) and phosphate buffer (0.01 M). The PO activity was measured colorometrically with the

help of spectrophotometer by taking absorbance at 470 nm at an interval of 2 min. The solution devoid of plant extract served as a blank [27].

Analysis of PPO Activity

Plant extract was mixed with 1.5 ml of sodium phosphate buffer (0.1 M) at 6.5 pH and 200 µl of catechol (0.1 M). The PPO activity was measured with the help of spectrophotometer by taking absorbance at 410 nm [27].

Analysis of PAL Activity

The plant extract (100 µl) was mixed with 0.1 M borate buffer (1.15 ml) at pH 8.8 and 10 mM L-phenylalanine (1 ml). The homogenate was placed in water bath at 40 °C for 60 min. The reaction was stopped by adding 250 µl of hydrochloric acid (5 N). The trans-cinnamic acid generated from L-phenylalanine was quantified spectrophotometrically at 290 nm to evaluate PAL activity [27].

Estimation of Total Phenolics

Total phenolics were quantified according to Zieslin and Ben-Zaken [28]. For this purpose, 1 g plant sample was extracted at 70 °C with 80% methanol (10 ml) for 15 min. Then 1 ml of this extract was homogenized with 5 ml distilled water and 1 N Folin-Ciocalteu reagent (250 µL) at room temperature. By taking gallic acid as blank, the quantity of phenolics in mixture was estimated spectrophotometrically at 725 nm.

Evaluation of Growth Promoting Attributes of Screened Rhizobacteria

The ACCD activity of screened rhizobacteria was analyzed according to Penrose and Glick [29]. While, phosphate solubilization, siderophore production and auxin synthesis potential of screened bacteria was analyzed according to Mehta and Nautiyal, Schwyn and Neilands and Nautiyal [30-32] respectively.

Evaluation of Screened Rhizobacteria Under Field Conditions

For field trial, split plot design having 5 replicates for each treatment was used. The experimental plot was divided into beds having 3 x 2 m² sizes. After priming *brassica* seeds with bacterial inoculum, these were sown and pathogen was applied after 25 days of sowing. The data regarding plant growth attributes was evaluated after 90 days of sowing.

Preparation of Peat Moss Based Inoculum of Bacterial Inducers

The peat moss was sterilized with the help of autoclave and inoculated with inoculum of screened rhizobacteris. For single bacterial inoculation, 100 g sterilized peat moss was inoculated with 50 ml of bacterial. For mixed bacterial formulation 100 gm sterilized peat moss was inoculated with 25 ml of both bacterial inoculums.

Statistical Analysis

The differences between the values obtained were estimated by performing one-way analysis of ANOVA and DNMRT at a significance level of 0.05 in DSASTAT software. Each trial was conducted for 5 biological repeats and the values represented in table's exhibit the average values of 5 replicates.

RESULTS

Potential of Bacterial Spp. against Alternaria Leaf Spot Disease of *B. Campestris*

The aim of current study was to screen some native bacterial spp. having potential to induce systemic resistance in *B. campestris* against *Alternaria* leaf spot caused by *A. brassicae*. In case of greenhouse experiment, typical symptoms of *Alternaria* leaf spot were observed on pathogen challenged plants. In general, the rhizobacteria inoculated plants challenged with pathogen showed delay in leaf spot symptoms, to some extent, as compared with pathogen control plants. However, *B. megaterium* ZMR-6 and *P. fluorescens* RB4 inoculated plants exhibited reduced disease index and better control effect (**Table 1**). Therefore, *B. megaterium* ZMR-6 and *P. fluorescens* RB4 were screened for downstream experimentation.

Elucidation of Biochemical Basis of Induced Resistance

During present study, inoculation of bacterial spp. helped *B. campestris* plants to synthesize significantly higher quantity of phenolics, PPO, PAL and PO in contrast to pathogen control (**Table 2**). It was observed that *B. megaterium* ZMR-6 and *P. fluorescens* RB4 enhanced phenolics level up to 62.28% and 49.16% respectively as compared with sterilized water control. Similarly, an improvement of 38.45%, 52.83% and 49.95% was observed in PPO, PAL and PO levels by inoculation of *B. megaterium* ZMR-6 as compared to sterilized water control.

Table 2. Effect of Bacterial spp. on elicitation of defense related biochemicals in *Brassica campestris* plants; Values are mean \pm standard deviation (n=3). Different letters represent significant difference at (P \leq 0.05) according to ANOVA and DNMRT, IOUC=Increase over untreated control.

Bacterial spp.	Phenolics ($\mu\text{g/h/gfw}$)	% IOUC	PPO Activity ($\mu\text{g/h/gfw}$)	%IOUC	PO Activity ($\mu\text{g/h/gfw}$)	%IOUC	PAL Activity ($\mu\text{g/h/gfw}$)	% IOUC
<i>Acinetobacter</i> sp. 334	1.51 \pm 0.06f-h	16.51 \pm 2.67j	5.46 \pm 0.51e-g	25.67 \pm 1.52f	1.05 \pm 0.15e-g	19.61 \pm 1.19g	2.01 \pm 0.39bc	04.37 \pm 0.82fg
<i>Acinetobacter</i> sp. CS9	1.76 \pm 0.07d-f	32.76 \pm 2.25fg	5.92 \pm 0.59bc	32.48 \pm 2.07c-e	1.23 \pm 0.08cd	35.21 \pm 2.53d	2.09 \pm 0.64bc	16.04 \pm 2.46e
<i>Aminobacter aminovorans</i> 374	1.73 \pm 0.09d-f	30.87 \pm 1.91gh	5.54 \pm 0.62e-g	35.55 \pm 4.48e	1.21 \pm 0.18d	30.98 \pm 2.18e	2.05 \pm 0.42bc	07.29 \pm 1.04f
<i>Bacillus fortis</i> 162	1.82 \pm 0.10c-e	39.14 \pm 2.92e	5.42 \pm 0.37f-i	24.53 \pm 2.43f	1.22 \pm 0.13d	30.78 \pm 2.51e	2.14 \pm 0.08b	12.45 \pm 2.03e
<i>Bacillus megaterium</i> ZMR-6	2.14 \pm 0.09c	53.47 \pm 4.52c	6.89 \pm 0.72ab	39.56 \pm 3.55c-e	1.41 \pm 0.12a	52.70 \pm 3.44a	3.01 \pm 0.36a	54.87 \pm 4.63b
<i>Bacillus subtilis</i> 170	2.06 \pm 0.15cd	48.82 \pm 2.50cd	5.12 \pm 0.60i	17.54 \pm 2.07g	1.09 \pm 0.08e-g	18.58 \pm 3.49gh	2.04 \pm 0.74bc	06.92 \pm 1.14f
<i>Bacillus subtilis</i> 189	2.01 \pm 0.08cd	46.17 \pm 2.36ef	6.02 \pm 0.70d-f	42.83 \pm 2.30cd	1.24 \pm 0.67cd	39.43 \pm 2.43c	2.64 \pm 0.13ab	43.71 \pm 6.53d
<i>Bacillus thuringiensis</i> 199	1.67 \pm 0.11e-g	28.19 \pm 2.42h	5.78 \pm 0.82fg	38.46 \pm 3.69de	1.07 \pm 0.07e-g	18.68 \pm 1.08gh	1.94 \pm 0.71bc	01.46 \pm 0.13g
<i>Bordetella pertussis</i> 263	1.58 \pm 0.14gh	20.86 \pm 1.92ij	5.19 \pm 0.61gh	28.83 \pm 1.84ef	1.15 \pm 0.26de	22.82 \pm 1.65fg	2.090 \pm 0.28bc	4.53 \pm 0.56g
<i>Burkholderia capacia</i> 337	1.80 \pm 0.12c-e	37.68 \pm 3.82ef	5.85 \pm 0.32d-g	29.16 \pm 3.27f	1.18 \pm 0.07d-f	25.86 \pm 1.92f	2.79 \pm 0.76ab	48.72 \pm 3.58c
<i>Burkholderia cepacia</i> CS8	2.26 \pm 0.09b	69.26 \pm 3.53a	6.28 \pm 0.53cd	43.94 \pm 2.82c	1.06 \pm 0.13fg	16.52 \pm 1.50gh	2.87 \pm 0.11a	48.20 \pm 2.68c
<i>Enterobacter</i> sp. CS2	1.83 \pm 0.08c-e	39.87 \pm 2.24e	6.53 \pm 0.72bc	55.98 \pm 4.67b	1.17 \pm 0.06d-f	25.80 \pm 2.83f	2.64 \pm 0.28a	43.42 \pm 3.05d
<i>Microbacterium lacticum</i> 261	1.79 \pm 0.08d-f	36.95 \pm 2.63ef	5.04 \pm 0.61hi	18.01 \pm 2.27g	1.07 \pm 0.19g	14.76 \pm 1.47h	2.17 \pm 0.31b	13.14 \pm 2.21e
<i>Pseudomonas fluorescens</i> 083	1.60 \pm 1.05e-g	23.08 \pm 1.05i	6.05 \pm 0.95c-e	45.88 \pm 5.36c	1.28 \pm 1.05bc	40.26 \pm 1.05c	2.01 \pm 0.26bc	06.15 \pm 1.26f
<i>Pseudomonas fluorescens</i> RB4	2.19 \pm 0.09b	65.15 \pm 4.28b	7.05 \pm 0.88a	68.59 \pm 9.21a	1.34 \pm 0.09b	47.59 \pm 3.82b	3.18 \pm 0.51a	62.45 \pm 7.39a
Sterilized distilled water	1.49 \pm 1.05gh	11.40 \pm 1.05k	5.34 \pm 0.24g-i	28.34 \pm 2.75f	1.08 \pm 1.05fg	15.49 \pm 1.05gh	1.97 \pm 0.17bc	04.65 \pm 0.85fg

Growth Promoting Attributes of Screened Bacteria

Results for growth promoting attributes exhibited that *B. megaterium* ZMR-6 was positive for ACCD activity while *P. fluorescens* RB4 was negative for this property. The both screened rhizobacteria showed capability of phosphate solubilization, siderophore production and auxin synthesis.

Field Experiment

Likewise greenhouse experiment, the screened bacterial spp. showed excellent results against *Alternaria* leaf spot disease under field trials. Co-inoculation of *B. megaterium* ZMR-6 and *P. fluorescens* RB4 proved more successful in management of *Alternaria* leaf spot disease as compared to individual application of bacterial sp. The screened rhizobacteria also improved growth and yield along with protecting *B. campestris* plants from leaf spot disorder during field experiments. Bacteria inoculated plants demonstrated significant improvement in plant height and total weight in contrast with un-inoculated control and pathogen treated plants. Co-inoculation of both bacterial spp. endorsed plant length up to 34% and 25% under I and II experiment, respectively. Similarly, *B. campestris* plants primed with bacterial spp. showed significantly higher number of pods. Thus current observations favor the use of PGPR to improve growth and manage disease of plants under field conditions.

DISCUSSION

Different beneficial bacteria and fungi may protect plants against biotic and a biotic stress [33-35] has reported that phenolic compounds protect plants from different pathogens. The screened bacterial inducers enhanced production of phenolic compounds (Table 3). Akram and Anjum [23] have demonstrated involvement of antioxidant enzymes (PAL, PPO, PO) in disease resistance. Some other researchers have also revealed role of these enzymes in plant disease resistance [36]. These defense related enzymes play a key role in phenylpropanoid pathway of plants which improve plant vitality and vigor. These enzymes also improve disease resistance in plants by degradation of pectolytic enzymes synthesized by disease causing fungi and bacteria [37,38]. Van Loon [39]

found that defense related enzymes increase the formation of lignin which acts as a barrier against pathogen infection. Ryals et al. [40] observed involvement of PAL in the phenylpropanoid pathway. PAL is also play a role in formation of lignin and flavonoids which restrict pathogen infection in plants [41]. PO helps in deposition of polysaccharide along with lignifications and suberization and lignification of plants which reduce the chances of disease development [42,43]. PPO helps in formation of antimicrobial compounds which enhance systemic resistance in plants against disease causing agents [44,45].

Table 3. Potential of selected bacterial spp. on Alternaria leaf spot management in Brassica campestris under field conditions; Values are mean ± standard deviation (n=3). Different letters represent significant difference at (P ≤ 0.05) according to ANOVA and DNMR. BM=B. megaterium ZMR-6, PF=fluorescens RB4, PC=Pathogen Control, UC=Untreated Control.

Treatments	Experiment-I		Experiment -II	
	Disease index	Control effect	Disease index	Control effect
	(%)	(%)	(%)	(%)
BM	35.72 ± 3.16c	56.14 ± 5.42b	26.75 ± 2.34bc	59.14 ± 4.32b
PF	43.51 ± 4.64b	45.52 ± 4.63c	30.26 ± 3.14b	61.16 ± 4.53b
BM ± PF	21.94 ± 3.92d	64.43 ± 8.61a	18.32 ± 1.23d	73.68 ± 6.24a
PC	79.38 ± 8.61a	ND	74.65 ± 05.43a	ND
UC	ND	ND	ND	ND

During current study screened bacteria induced improved activity of defense related biochemicals and enzymes in brassica plants resulting enhanced systemic resistance [46,47]. Since each bacterial isolate was obtained from different resource, therefore each bacterium showed difference in disease reduction [33,48]. Our results for improved disease resistance in case of combined application of screened bacteria are in accordance with the findings of [49]. Our results regarding higher production of defense related biochemicals and enzymes in combined bacterial application confirm findings of Raupach and Kloepper [50]. Jetiyanon and Kloepper [51] also reported improved production of defense related metabolites and reduced disease level in plants inoculated with PGPR consortium.

Nihorimbere et al. [52] found enhanced growth in plants assisted with disease reducing microbes. Similarly, Bacon and Hinton (2002) [53] revealed improved plant growth under the influence of bacterial inducers. Our study also showed improved plant growth, biomass production and pod formation in plants assisted with bacterial inducers. PGPR synthesize growth promoting hormones which may improve plant growth [54]. Glick et al. [55] reported that ACCD producing PGPR lowers ethylene production resulting improved biomass production in plants under stress. On the other hand some other scientists observed that siderophore producing rhizobacteria improve iron availability to plants which in return improve growth of plants [56-58]. Our results are also in congruent with findings of Adhikari et al. [52]. Bacon and Hinton [47] also found role of bacterial inducer in improvement of growth in co cultivated plants (Table 4).

Table 4. Effect of bacterial spp. on growth attributes of Brassica campestris under field conditions; Values are mean ± standard deviation (n=3). Different letters represent significant difference at (P ≤ 0.05) according to ANOVA and DNMR. BM=B. megaterium ZMR-6, PF=fluorescens RB4, PC=Pathogen Control, UC=Untreated Control.

Treatments	Experiment 1				Experiment 2			
	Plant height	Total weight (g)		Number of	Plant Height	Total weight (g)		Number of
	(cm)	Fresh	Dry	Pods	(cm)	Fresh	Dry	Pods
BM	31.42 ± 1.94b	108.34 ± 7.54bc	12.45 ± 2.32ab	17.35 ± 1.74bc	36.07 ± 3.26b	123.54 ± 7.82b	19.36 ± 2.43b	16.45 ± 2.35c

PF	28.53 ± 2.17bc	97.48 ± 6.46c-e	9.82 ± 1.68b-d	19.63 ± 2.04b	32.76 ± 2.83bc	107.16 ± 6.43c	14.89 ± 1.96c	13.48 ± 2.62cd
BM ± PF	36.86 ± 3.24a	117.82 ± 8.63ab	14.25 ± 1.53a	25.81 ± 3.46a	39.45 ± 4.05ab	148.94 ± 8.76a	24.68 ± 3.14a	24.13 ± 1.97a
PC	17.18 ± 1.72e	48.37 ± 4.25g	5.94 ± 0.106de	08.16 ± 0.82d	19.82 ± 2.46e	63.56 ± 06.42d	08.69 ± .93e	05.65 ± 1.05e
UC	25.68 ± 2.36cd	89.68 ± 06.96ef	7.92 ± 01.36cd	18.73 ± 2.81b	29.86 ± 2.92cd	119.53 ± 9.11b	12.82 ± 1.34cd	18.94 ± 1.58bc

CONCLUSION

The present study exhibited that *B. megaterium* ZMR-6 and *P. fluorescens* RB4 are capable to induce systemic resistance and reduce *Alternaria* leaf spot disease in *brassica*. This improved disease resistance may be attributed to enhance production of defense related biochemicals and enzymes in plants under the influence of these rhizobacteria. Moreover, the growth promoting characteristics of *B. megaterium* ZMR-6 and *P. fluorescens* RB4 improve growth, biomass production and yield of associated *brassica* plants in an environmentally safe way.

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