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Minimizing the allelopathic effects of *Rosmarinus officinalis* L. on intercropped wheat by plant growth promoting bacteria

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ABSTRACT

We assessed the potential of plant growth promoting bacteria (PGPB) of *Pseudomonas* spp. and *Bacillus* spp. to minimize the allelopathic effects of rosemary (*Rosmarinus officinalis* L) on intercropped wheat (*Triticum astivum* L) plants. Blotter paper test revealed three superior strains (Pf-19, Pf-37 and BS-56) among the 10 selected PGPB strains. Rosemary leaf extract reduced the growth of intercropped wheat plants. The application of the selected bacterial strains, however, significantly reduced the allelopathic effects and stimulated the seed germination and seedling vigour. Among the different treatments, combined treatment of two PGPBs (Pf-37 + Bs-56) showed best results to promote wheat growth and reduce the allelopathic effects of rosemary on wheat seedlings.

Key words: Allelopathic effects, *Bacillus subtilis*., catalase, PGPB, peroxidase, *Pseudomonas fluorescens*, *Rosmarinus officinalis*, Rosemary.

INTRODUCTION

Rosemary (*Rosmarinus officinalis*) is an important medicinal plant cultivated in Uttarakhand region (Fig.1). It is a household culinary spice for flavouring foods (3,4). Being perennial, rosemary is harvested upto 10-12 years. Interplanting of agricultural crops with medicinal plants is practised to increase the farmer's income. However, due to its allelopathic effects, intercrops such as wheat (*Triticum aestivum*), amaranthus (*Amaranthus virdis*), finger millet (*Eluecine indica*) are adversely affected, when grown in the vicinity of rosemary (5,9).

Figure 1. *Rosemarinus officinalis* plants under field conditions.

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Allelochemicals released from plants affect the growth of other plants in its vicinity. However, studies suggest that the use of plant growth promoting soil bacteria can help in the management of abiotic and biotic stresses including allelopathy (6,14,20,24). Rhizobacteria such as *Pseudomonas fluorescens* and *Bacillus subtilis* promote plant growth and may be used in sustainable agriculture to promote plant growth and suppress plant diseases (12,25). This study aimed to examine the role of plant growth promoting *P. fluorescens* and *B. subtilis* in minimizing the allelopathic effects of rosemary on wheat.

MATERIAL AND METHODS

The studies were conducted in November-December 2016 and 2017 in the Department of Seed Science & Technology, HNB Garhwal University, Srinagar, Garhwal **[**Latitude and longitude are 30^{o}22'N and 78^{o}80' E, Elevation from mean sea level : 560 m, Mean annual rainfall : 1371 mm.].

Preparation of leaf extracts

Fresh leaves of 2-3 years old rosemary plants were harvested, washed with sterile distilled water and dried in shade (5-7 days). The shade dried leaves were powdered, ground and the allelochemicals from the leaves were extracted in sterile distilled water by the cold extraction method (27). For this, 25 g rosemary leaf powder was suspended in 100 ml distilled water in 250 ml conical flasks**.** These flasks were kept on a mechanical shaker at 25°C at 100 rpm for 48 h**.** Afterwards, the solution was filtered through Whatman No.1 filter paper and the filtrate was used for further experiments. Three doses (2 %, 5 % and 10 %) of rosemary leaf extract (based on leaves weight) were used to evaluate its effects on wheat seed germination and seedlings growth. From preliminary studies (data not shown), 10 % leaf extract reduced the seed germination and seedlings growth of wheat and was hence, selected for further studies

Bacterial strains

Ten pre-characterized plant growth promoting (PGP) strains of *Pseudomonas* spp. and *Bacillus* spp. (5-each) were used. These strains are well characterized for PGP and biocontrol potential (13). The strains were obtained from the repository of the Microbiology laboratory, College of Forestry, Ranichauri and were maintained on King's medium B (18) and Nutrient agar medium, respectively and were sub-cultured when required.

Talc formulation

Talc-based formulations of all 10-bacterial strains were prepared as per the method of Kloepper and Schroth (19). All strains were individually grown in 250 ml Erlenmeyer flasks containing pre-sterilized KB broth or nutrient broth (100 ml in each flask), respectively. The flasks were then incubated at 28±2^{o}C on an incubator shaker at 120 rpm for 48-72 h to get a bacterial population of about $3x10^9$cfu ml^{-1}. The bacterial suspension was then mixed with pre-sterilized talc powder (1:2) containing 1 % carboxymethyl cellulose to get a final cfuof at least $1x10^7g^{-1}$ in the formulation**.** The formulation was air-dried, packed in pre-autoclaved poly bags and stored at -20^{o}C until further use within 3 months.

Seed bacterization

Wheat variety ‘VL 616’ was obtained from the Department of Crop Improvement, College of Forestry, Ranichauri. Seeds were inoculated (bacterized) with talc formulations (@ 8g kg^{-1} seeds). For this, healthy seeds (non-diseased, unbroken) were selected, weighed and moistened with sterilized distilled water. Talc formulation of the respective bacteria was added to the respective set and mixed gently to form a fine coating on the seeds. The inoculated seeds of all sets were then air-dried for 4-6 h. Seeds in control were treated only with fresh talc.

Selection of putative bacterial strains

Initially, 10-bacterial cultures were used to assess their potential in minimizing the allelopathic effects of rosemary leaf extracts using the blotting paper test. Blotting papers (18 cms d) were pre-soaked in 10 % rosemary leaf extract (10 ml), except for the control set, which was treated with sterile distilled water. Similarly, seeds were bacterized with individual bacterial formulation (@8 g kg^{-1} seeds), whereas, the control seeds were treated with sterile water only. Twenty seeds were placed in separate Petriplates (18 cm dia.) containing the moist blotting paper. All Petriplates were then placed in seed germinator at 25±1 ^{o}C and germination was recorded daily until the complete germination. Ten days after germination, 10-seedlings per Petri plate were taken to record growth parameters (root length, shoot length, fresh weight, dry weight and seedling vigour). Dry weight of seedlings was recorded after drying the seedlings at 72 ^{o}C for 12 h. Seedling vigour was calculated by using the following equation (17).

Seedling vigour = % Germination X Seedling length

Pot Experiment

Pot trial (4-weeks) was done at Srinagar, Garhwal during November-December 2016 and 2017. In 2016 it was sown on November 7 and harvested on December 6, while in 2017, it was sown on November 11 and harvested on December 10.Two strains of *P. fluorescens* Pf-19 and Pf-37 and one *B. subtilis* strain Bs-56 was selected based on blotter paper test and were used as candidate organisms for pot (15 cm dia and 15 cm depth) assay to determine their potential to minimize the allelopathic effects of rosemary leaf extracts on wheat. Soil, sand and FYM mixture (2:1:1) was used as growing medium (3 kg pot^{-1}).

Table 1. Details of treatments used in pot experiment

Treatment	Treatment details
Control (C-1)	Soil and seeds without any treatment
Negative control (C-2)	Soil+10 % rosemary leaf extract but seeds received no treatment
T-1	Soil+10 % rosemary leaf extract and seeds treated with Pf-19 @ 8g/kg seeds
T-2	Soil+ 10 % rosemary leaf extract and seeds treated with Pf -37 @ 8g/kg seeds
T-3	Soil+ 10 % rosemary leaf extract and seeds treated with Bs-56 @ 8g/kg seeds
T-4	Soil+ 10 % rosemary leaf extract and seeds treated with Pf -19 + Pf -37 @ 8g/kg seeds (4g each)
T-5	Soil+10 % rosemary leaf extract and seeds treated with Pf -37 + Bs-56 @ 8g/kg seeds (4 g each)
T-6	Soil+10 % rosemary leaf extract and seeds treated with Pf -19 + Bs-56 @ 8g/kg seeds (4g each)
T-7	Soil + 10 % rosemary leaf extract and seeds treated with Pf -19 + Pf -37 + Bs-56 @ 8g/kg seeds (2.6 g each)

All the treatments were used in triplicates. Data presented in the paper is average of these triplicates.

The mixture was sterilized by autoclaving before filling into the pots. Details of treatments are given in Table 1. The wheat variety 'VL 616' seeds were bacterized with talc formulation (@ 8g kg^{-1} seed) and 15 seeds were sown in each pot.

To test alleopathic effects, 25 ml rosemary leaf extract (10 %) was added to each pot except the control. In control, 25 ml water was added. The pots were kept in screen house and 3 days after sowing were irrigated daily with 25 ml water. Data for germination (%) was recorded daily, until the complete germination in all pots. However, the data for root length, shoot length, fresh weight and dry weight were recorded one month after sowing. Effects on various plant growth parameters were recorded as per ISTA rules (17).

Total protein estimation

To estimate total protein in wheat seedlings, 1 g of seedlings from each treatment were taken and ground with liquid N_2 using pre-chilled mortar and pestle. Immediately after grinding, 5.0 ml of extraction buffer containing 0.14M Tris-HCl buffer (pH-6.8), 4 % SDS, 2 % 2-mercaptoethanol, 20 % glycerol, 0.02 % bromophenol blue was added and mixed gently. The extract was then centrifuged at 10000 rpm for 10 min and the supernatant was collected. Absorbance of these samples was recorded at 540 nm by UV-Vis spectrophotometer. Concentration of protein in samples was calculated against the standard curve developed using bovine serum albumin (26)

Enzyme assays (Specific activity)

For Enzyme assays, fresh seedlings (from each treatment), were uprooted on 2nd, 4th and 6th day after the emergence of radicle. One g of fresh seedling from each treatment was taken and crushed in 10 ml extraction buffer containing 100mM Tris (pH-7.5), 1mM EDTA, 0.5 % Triton X-100, 1mM dithiothreitol (DTT) and 2 % polyvinyl pyrrolidone (PVP). The suspension was centrifuged for 10 min at 10,000 rpm at 4 °C. The supernatant was used to determine the peroxidase (POX) and catalase (CAT) activity.

Guaiacol peroxidase (POX) was determined spectrophotometrically according to Chance and Maehly (7) with slight modifications. Briefly, the reaction mixture was prepared containing 50 mM sodium acetate buffer (pH 5.5), 92 mM guaiacol, 18 mM H_2O_2 and 5 µl of enzyme extract. The reaction was monitored at 470 nm and the reaction rate was calculated from the coefficient of absorbance for tetraguaiacol of 25.5 cm^2 μmol^{-1}.

Catalase (CAT) was measured by the method of Aebi (1). The reaction mixture contained 1.5 ml of 100 mM potassium phosphate buffer (pH-7) and 0.05 ml enzyme extract. The final volume was made to 3.0 ml by adding sterile distilled water. The reaction was started by adding 0.5 ml of 75 mM H_2O_2 and the decrease in absorbance was recorded at 240 nm after one min. Enzyme activity was determined by calculating the rate of disappearance of H_2O_2 by at 240 nm and expressed in µmol ml^{-1} min^{-1}permg^{-1} ml^{-1}.

Statistical Analysis

Data in this paper is the mean of 2-years**.** The data was statistically analyzed by using one-way ANOVA to calculate the magnitude of the F value.

RESULTS AND DISCUSSION

The rosemary leaf extract (10 %) had allopathic effects on wheat and affected the seed germination (Table 2, Fig. 2A). The effect was prominently seen in negative control (C-2) with 42.50 % inhibition in germination than in control (C-1). Allelochemicals are known to adversely affect the seed germination of sensitive plant species (8,24). Essential oil from rosemary is also allelopathic to seed germination and seedling growth of wheat (5).

Our results suggested that seed treatment with *Pseudomonas* and *Bacillus* strains reduced the allelopathic effects of rosemary in wheat (Table 2). Treatment T-5 (Pf-37 + Bs-56) was found best as this significantly increased all plant parameters. However, remaining treatments [T-1 (Pf-19), T-4 (Pf-19 + Pf-37) and T-6 (Pf-19 + Bs-56)] increased most of the parameters, except the speed of germination and seed germination (%) of wheat, whereas, T-7 showed non-significant effects. Combination of Pf-37 and Bs-56 (T-5) showed significant increase (~180 %) in all plant parameters over negative control (Fig. 2B). In laboratory experiments, leachates from rosemary suppressed the growth of plants [*Schizachyrium scoparium*, *Leptochloadubia*, *Eryngium cuneifolium* and *Hypericum cumulicola*] (11,15) but many studies suggested that seed treatment with PGPR promoted the plant growth. Kumar *et al* (19) evaluated the synergistic effects of two PGPR namely *P. putida* NBRIRA and *B. amyloliquefaciens* NBRISN13 in chick pea**.** They observed that these bioagents enhanced the germination and growth parameters in chickpea. Mishra and Nautiyal (24) evaluated the role of *P. putida* NBRIC19 in alleviating the biotic stress of *Parthenium hysterophorus* in wheat. They found that seed treatment with *P. putida* NBRIC19 effectively increased the root length, shoot length and dry weight in wheat by 52.29, 28.73 and 76.31 %, respectively, over control.

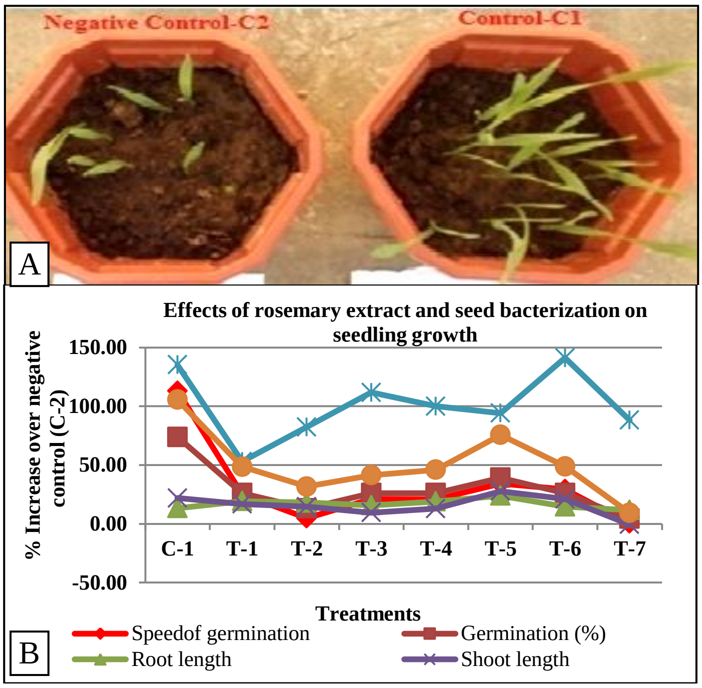

Figure 2. Allelopathic effects of rosemary on wheat and its management by seed inoculation with selected bacterial strains.

A. Effects of rosemary leaf extract on germination and growth of wheat. **B.** Percent increase in seedling growth parameters over negative control (C-2). For details of treatments, see Table 1.

Since, catalase and peroxidase are known as protective enzymes, we examined their response in wheat seedlings grown under allelopathic conditions. However, bacterization of wheat seeds with growth promoting bacteria was done to reduce the allelopathic effects. The Enzyme assays revealed significant differences in the specific activity of both the enzymes (Table 2). However, on the second day after the emergence of radicles, CAT specific activity was higher in the negative control (C-2) than in control (C-1) (Table 3). Further, there was gradual decrease in CAT activity in negative control on 6^{th} day. However, maximum CAT activity was recorded in treatment T-5 followed by T-4 on 2^{nd} day but declined on the 6^{th} day. Similarly, maximum POX activity was recorded in treatment T-7 followed by T-4 on 2^{nd} day but then declined on the 4^{th} and 6^{th} day. The same pattern in the specific activity of both the enzymes was observed in all treatments. However, T-7 consistently showed higher activities for both enzymes even on 6^{th} day (Table 3). This indicates that plants in this treatment could not come out of the stress and therefore, consistently showed higher enzyme activity. Results of enzyme assay suggested that the specific activity of both enzymes was affected significantly by seed treatment with selected bioagents and reduced the stress caused by rosemary leaf extract.

Table 2. Effects of seed treatment on germination and seedling growth of wheat grown in soil amended with rosemary extract

Treatment	Speed of germination[a]	Germination (%)	Root length (cm)	Shoot length (cm)	Dry weight[b] (g)	Seedling vigour[c]
C-1	2.39*	88.88*	13.57*	16.73*	0.13*	2702.06*
C-2	1.12	51.10	11.98	13.73	0.05	1314.66
T-1	1.40	64.44	14.28*	16.04*	0.08*	1952.62*
T-2	1.17	57.77	14.15*	15.72*	0.09*	1730.20
T-3	1.36	64.44	13.87*	15.03	0.11*	1858.78*
T-4	1.35	64.44	14.25*	15.49*	0.11*	1918.62*
T-5	1.50*	71.10*	14.85*	17.54*	0.14*	2308.57*
T-6	1.45	64.44	13.73*	16.62*	0.13*	1956.15*
T-7	1.13	53.32	13.38	13.64	0.10*	1436.49
CD at 5 %	0.37	14.09	1.41	1.34	0.04	462.25

Data presented in the table is average of two years.
Values given in the table are mean of three replicates. Rosemary leaf extract used in the experiment was of strength 10 %. * Data is significant at 5 % level of significance.
[a] Speed of germination: $n_1/d_1 + n_2/d_2 + n_3/d_3 + \dots\dots\dots\dots$, Where, n : number of germinated seeds, d : number of days. [b] dry weight was recorded after drying the seedlings at 72 ^oC for 12 h.[c] Seedling vigour : % Germination x seedling length.

Biotic and abiotic stresses induce the formation of reactive oxygen species (ROS) affecting the plant catabolic activity, which causes oxidative damage to membrane, lipids, proteins and nucleic acids (31). Under stress conditions, plant cells and subcellular systems are protected from the cytotoxic effects of the active oxygen radicals by the antioxidant enzymes [catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GTR) (2)]. These antioxidant enzymes act as the eliminator of H_2O_2 from the plants (28,29,30) to support the growth and survival of the plant under stress conditions.

Table 3. Effects of treatments on specific activity of catalase and peroxidase activities on various days after radical emergence.

Treatments	Specific activity* of Catalase (µmol mg^{-1} min^{-1})			Specific activity* of Peroxidase (µmol mg^{-1} min^{-1})		
	2^{nd} day	4^{th} day	6^{th} day	2^{nd} day	4^{th} day	6^{th} day
C-1	0.32	0.33	0.29	1.27	1.18	1.00
C-2	0.43	0.46	0.40	1.54	1.35	1.23
T-1	0.48**	0.54**	0.37	1.36**	1.22**	1.01
T-2	0.47**	0.54**	0.33	1.33**	1.19	1.00
T-3	0.47**	0.60**	0.34	1.49**	1.31**	1.11**
T-4	0.52**	0.48**	0.32	1.80**	1.46**	1.22**
T-5	0.56**	0.58**	0.35	1.33**	1.19	1.00
T-6	0.48**	0.58**	0.35	1.42**	1.25**	1.04**
T-7	0.47**	0.45**	0.47**	1.89**	1.72**	1.41**
CD at 1 %	0.03	0.02	0.04	0.04	0.04	0.04

Data presented in the table is average of two years.
Values are means of three replicates. Plants were grown in the soil amended with rosemary extract (10 %), ** values are significant at 1 % level comparing with C-2, *Specific activity: Enzyme activity in solution (µmol ml^{-1} min^{-1}) / Protein concentration in sample (mg^{-1} ml^{-1})

CAT activity plays an important role in scavenging the reactive oxygen species (ROS) and protects the wheat plants against stress (10). Higher CAT activity occurred in okra plants inoculated with *Enterobacter* sp. UPMR18, *B. megaterium* UPMR2 and combined PGPR application than control under salt stress (14). Similarly, Minaeva and coworkers (23) reported increased peroxidase activity in roots of wheat plants treated with *Pseudomonas* strains GS4 and PhS1. These plants showed less disease development than untreated plants, when infested with *Bipolaris soroikiniana* fungus.

On the 6^{th} day after radical emergence, a decrease in the specific activity was observed in different treatments (Table 3). This suggested that the plants inoculated with the *P. fluorescens* and *B. subtilis* felt less stress on the 6^{th} day. Several genera of soil bacteria, including *Pseudomonas* and *Bacillus* species, stimulate root proliferation and protect from biotic and abiotic stresses by effective rhizosphere colonization (22,32,33,34). Mishra and Nautiyal (24) reported the lower activity of CAT and APX (Ascorbate peroxidase) under the biotic stress of *Parthenium* in wheat by biological seed treatment. Studies suggest significantly lower activities of antioxidant enzymes [Ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX)] in treated plants than control under stress, indicating that treated seedlings felt less stress than untreated plants (14,21). Our study suggested that the combination of Pf-37 and Bs-56 Plant roots communicate with soil microbes in a sophisticated manner through chemical communication to attract beneficial microbes including plant growth promoting bacteria and results in priming of defense, or induced resistance in the plant host (22) was most effective in reducing the allelopathic effects of rosemary on wheat.

CONCLUSIONS

The treatment of wheat seeds with *P. fluorescens* (Pf-19, Pf-37) and *B. subtilis* (Bs-56) reduced the allelopathic effects of rosemary on wheat. Best performance was found with combinations of Pf-37 + BS-56. Additionally, results of enzyme assay suggested that the specific activity of both enzymes was affected significantly by seed treatment with selected bioagents and reduced the stress caused by rosemary leaf extract. Since the combination of two PGPBs can reduce the allelopathic effects of rosemary, this may therefore be recommended to farmers interested in co-cultivation or intercropping of wheat and rosemary after more research.

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