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EFFECT OF AQUEOUS SMOKE SOLUTION EXTRACTED FROM *Peganum harmala* AND THE BACTERIA ISOLATED FROM THE RHIZOSHPERE OF *Ipomoea aquatic* ON WHEAT GROWTH

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The combined effect of the plant derived aqueous smoke solution and the growth promoting rhizosphere bacteria on wheat was investigated in the present study. The bacterial strains (NCCP-549, 550, 551 and 552) were isolated from the rhizosphere of *Ipomoea aquatica* Forssk treated with the aqueous smoke solution derived from *Peganumharmala* L. Here we tested the isolated bacterial strains for their growth promoting effect on wheat (*Triticum aestivum* L.) alone or along with different dilutions of the smoke solution in water (1:100 and 1:500 (v/v)). A series of bioassays were performed both in *in vitro* on plates and *in vivo* in pots along with all the required controls. The bacteria were characterized biochemically and identified at molecular level as *Chryseobacterium*, *Acinetobacter* and *Bacillus* spp. The combined treatment of bacterial strains and the aqueous extract of smoke significantly increased seed germination, seedling and plant growth as depicted by the root/shoot length, plant weight, chlorophyll, carotenoid, nitrogen, and protein contents. The shoot weight increased up to 90 % by application of bacterial strains, alone or along with smoke solution as compared with control. Similarly, the chlorophyll, carotenoid, nitrogen and protein contents also increased up to 30-58 %, 54 %, 109 % and 109 % due to application of the treatments, respectively. The strain NCCP-550 along with the smoke solution (1:100) showed the maximum percentage increase in the different parameters. This research highlighted the possibilities of combined use of aqueous extract of smoke and the plant rhizosphere bacteria for improved growth and production of wheat.
Keywords: wheat; plant growth promotion; plant derived smoke solution; rhizosphere bacteria.

INTRODUCTION

Smoke is an airborne by-product of natural processes resulting in combination of solid, liquid and gaseous particles. Smoke includes a complex mixture of the visible products of burning containing the primary and secondary gaseous products. The aqueous solution can be prepared by passing smoke produced by partial burning of dried plant through water (Dixon *et al.*, 1995).The smoke was reported to have plant growth promoting effects because it contained different plant growth promoters (Chiwocha *et al.*, 2009).Furthermore, smoke also enhanced the plant growth by affecting the plant hormones (Van Staden *et al.*, 2000). It was also used to improve plant germination and break seed dormancy (Van Staden *et al.*, 2000). The underlying mechanisms have not yet been described clearly. However, it has been suggested that it may have an impact on the seed coat and embryo (Morris *et al.*, 2000). The quantity and composition of smoke may differ depending on the chemistry and plant species (Dixon *et al.*, 1995). The aqueous solution of smoke extracted from burning of *Peganum harmala* previously showed promotion of plant growth and seed germination (Khan *et al.*, 2014). Moreover, a number of compounds have been identified so far from the aqueous smoke solution playing roles in plant growth promotion and seed germination (Kamran *et al.*, 2017).

Application of beneficial bacteria in agriculture has increased globally. Plant growth promoting bacteria (PGPB) may promote the plant growth by increased availability of certain nutrients or by release of certain metabolites (Liu *et al.*, 2018). Plant growth promoting bacteria. may improve plant growth by using different mechanisms of action either directly and/or indirectly. The indirect mechanisms include opposition against plant pathogenic microorganisms by producing antibiotics, enzymes, siderophores, or pigments (Pal *et al.*, 2001). Direct growth promotion may be through solubilization of minerals, nitrogen fixation and/or hormones production such as indole acetic acid, cytokinins and

gibberellic acid that may enhance root length and plant growth (Gutiérrez *et al.,* 2003;Son *et al.,* 2006;
The present study was aimed to investigate the combined effect of the rhizosphere bacterial strains and plant derived smoke extract on germination and growthof wheat plants.

MATERIALS AND METHODS

***Phenotypic and biochemical characterization of the bacterial strains*:** Four bacterial strains i.e. NCCP-549, NCCP-550, NCCP-551 and NCCP-552 were isolated from the rhizosphere of *I. aquatica* treated with smoke solution derived from *P. harmala* using spread plate technique on nutrient agar medium. The isolates were phenotypically characterized by Gram staining, morphology, growth and biochemical characteristics including indole production, triple sugar iron reaction (TSI), oxidase test, catalase, methyl red and vogesproskauer, nitrate test, starch hydrolysis test, phenyl alalanine deaminase test, citrate utilization test and motility of organism (Verma *et al.*, 2012).
***Molecular identification of the bacterial strains*:** The 16S rRNA gene was sequenced for molecular identification of the bacterial strains (Juretschko *et al*., 1998). DNA extracted and amplified by colony-PCR using 9F and 1510R primers (Juretschko *et al.,* 1998). The amplicons were observed on 0.8% agarose gel electrophoresis (Juretschko *et al.*, 1998). The amplicons were sequenced by Macrogen service laboratory in Korea. Sequences were then analyzed using BLAST (blastn) program of the National Centre of Biotechnology Information (NCBI).
***Preparation of smoke solution*:** Stem and leaves of *P. harmala* L. were collected from Kohat region in the North West of Pakistan. The plant leaves were cleaned and semi-dried under shade for 15 days at room temperature. Aqueous extract of smoke was then prepared with some minor modification to the procedure previously reported (Dixon *et al.*, 1995). Briefly, plant material was smoked through distilled water until completely converted to ash; the solution was filtered and stored at 4°C. This concentrated aqueous smoke extract was diluted with water in ratios of 1:100 and 1:500 (v/v).
***Elemental analysis of the smoke solution*:** The plant derived smoke solution was analyzed for N, Mg, Fe, Cu, Mn, Ca, K, and Na. The Kjeldahl process was used to determine the concentration of Nitrogen (Anonymous, 1990). The Atomic Absorption Spectrophotometer was used to assess the quantities of Mg, Fe, Cu and Mn (Model Analyst 700, Perkin Elmer, USA) by making standard curves using AR grade solutions of the elements i.e. Mg, Fe, Cu and Mn. Concentration of Alkali metals including Ca, K and Na was found using Flame Photometer (Awan and Salim, 1997).
Effect of the smoke solution on the physiology, morphology and growth of bacterial isolates: The bacterial isolates were cultured on nutrient agar amended with aqueous smoke extract (0.5%) along with control without smoke solution. The morphology and physiology of the strains were observed after incubation for 3 days. The biochemical analyses of the bacterial strains were also conducted upon treatment with the smoke solution.
***Biopriming of seeds*:** Wheat seeds were collected from Barani Agricultural Research Station Kohat, Pakistan. The seeds were cleaned and surface sterilized in 5% NaOCl solution for 10 min and then washed with sterilized distilled water followed by drying in air at 25°C (Sauer and Burroughs, 1986). The seed priming was performed in 2% sucrose inoculated with the different bacterial strains (6 $x10^8$ CFU /ml) along with shaking at 200 rpm for 48 hr and then dried at room temperature in sterile conditions.
***Laboratory germination assays*:** For laboratory germination assays, Petri plates (9 cm) with two layers of moistened filter paper were used. The different treatments included a) only bacteria; b) bacteria plus aqueous smoke solution with different dilutions (1:100 and 1:500); c) only aqueous smoke solutions. The bacterial inocula used in the assays contained 6 $x10^8$ CFU /ml. Each plate containing 10 seeds was replicated thrice. The plates were moistened either with distilled water (5 mL) or with the respective smoke dilution. The bacterial strains (NCCP-549, NCCP-550, NCCP-551 and NCCP-552) were inoculated by seed biopriming as explained above. The Petri plates were incubated at 25°C in dark and the germination was noted after 24 h. Root and shoot lengths were recorded after 10 days of incubation.
***Greenhouse assays*:** The greenhouse assays were conducted in plastic pots with 1 kg soil per pot. The clayey loam soil was taken from the local fields of Kohat University of Science & Technology, Kohat Pakistan. The soil was then cleaned and autoclaved before use for the bioassays. The treatments used in these assays included a) only bacteria; b) bacteria plus aqueous smoke solution with different dilutions (1:100 or 1:500); and c) only smoke solution. Each treatment was repeated 3 times with 5 seeds in each replica. The bacterial inocula used in the assays contained 6 $x10^8$ CFU /ml. The pots were irrigated with 200 mL of distilled water or of the respective smoke solution every two days. The bacterial application (strains NCCP-549, NCCP-550, NCCP-551 and NCCP-552) was performed by seed biopriming as explained above. Plants were harvested after four weeks. The shoot length and fresh/dry weights were measured. The plants were then chopped and dried at 80°C in an incubator. The cell membrane stability (Jamil *et al.*,2012), chlorophyll and carotenoids contents (Lichtenthaler and Wellburn, 1983), and the total organic nitrogen and protein content (Pellett and Young, 1980) were also determined.
***Statistical analysis*:** The data were analyzed by analysis of variance (ANOVA) for significance and the means were compared using Fisher's least significant difference (LSD) tests.

RESULTS

***Characterization and identification of bacterial strains*:**
Morphological characteristics: The bacterial strains (NCCP-549, NCCP-550, NCCP-551 and NCCP-552) were characterized for colony characteristics including colony shape, elevation, margin and color. NCCP-549, NCCCP-550 and NCCP-551 had smooth circular convex shaped colonies while NCCP-552 had intricate colonies. Colony color of NCCP-549 to 552 was yellow, off white, off white, creamy off white, respectively. NCCP-550 and 551 were cocco-bacilli and the other two strains were rod shaped. NCCP-552 was the only Gram positive and motile strain.
***Biochemical characteristics*:** We obtained positive results for catalase and methyl red tests while negative for Voges-Proskaur test. Only NCCP-549 was positive for oxidase, indole and phenylalanine deaminase tests (Table 1). NCCP-550 and NCCP-552 were positive for starch hydrolysis. Similarly, NCCP-549 was the only isolate negative for citrate utilization and nitrate tests. NCCP-549 and NCCP-552 butt were in red color and slant were in yellow color, while NCCP-550 and NCCP-551 butt and slant both were in red color and all the isolates were negative for gas and hydrogen sulphide production.
***Molecular analysis*:** Based on 16S rDNA, the strain NCCP-549 had significant homology with *Chryseobacterium glum* (98% identical), the strain NCCP-552 to *Bacillustequilensis* (99.9% homology) and the strains NCCP-550 and NCCP-551 to *Acinetobacterpittii* (with homology of98% and 100%, respectively; Table 2). The 16S rRNA gene sequences of strains NCCP-549 to 552 were submitted in the GenBank under accession numbers as AB719399, AB719400, AB719401 and AB719402, respectively.
***Effect of the smoke on the physiology, morphology and growth of bacterial isolates*:** Supplementing smoke solution in the culture medium did not change the morphology or physiology of the bacteria under investigation.
***Elemental analysis of smoke solution*:** The smoke solution (extracted from *P. harmala*) was analyzed for different elements. It was found that smoke solution have Nitrogen, Calcium, Sodium, Magnesium, Iron, Copper, Manganese and Potassium at variable concentrations (Table 3).
***Germination percentage*:** Inoculation with strain NCCP-552 in combination with smoke (1:500) showed the highest %age germination after 24 h followed by the NCCP-550 along with smoke solution (1:100; Figure 1). The smoke solution in combination with bacterial inoculation generally had significantly higher germination percentages as compared to the treatment of bacteria alone. Overall, the germination percentage of the seeds increased significantly upon treatment with either smoke or smoke in combination with bacteria as compared to control or the bacteria alone. The bacteria alone had no impact on the seed germination percentages as compared to control except the strain NCCP-552.
***Effect of inoculation and smoke treatments on plant growth parameters using in vitro conditions*:** The shoot and root length of seedlings significantly increased by treatment with bacteria in comparison with the control (Figure 2 & 3).

Table 1. Biochemical characteristics of bacterial strains isolated from *Ipomoea aquatica* treated with smoke solution derived from *Peganum harmala.*

	BIOCHEMICAL TEST											
Bacterial strains	**OX**	**CIT**	**CAT**	**MR**	**VP**	**NT**	**IT**	**SHT**	**PDT**	**TSI (Slant/butt)**	**Gas**	**H_2S**
NCCP-549	+	-	+	+	-	-	+	-	+	Yellow/Red	-	-
NCCP-550	-	+	+	+	-	+	-	+	-	Red/Red	-	-
NCCP-551	-	+	+	+	-	+	-	-	-	Red/Red	-	-
NCCP-552	-	+	+	+	-	+	-	+	-	Yellow/Red	-	-

Note: OX(Oxidase test), CIT(Citrate utilization test), CAT(Catalase test), MR(Methyl red), VP(Voges-Proskauer test), NT(Nitrate test), IT(Indole test), SHT(Starch hydrolysis test), PDT(Phenylalanine deaminase test), TSI(Triple sugar Iron Test).

Table 2. Molecular identification of bacterial strains isolated from isolated from *Ipomoeaaquatica* treated with smoke solution derived from *Peganum harmala* using 16S rRNA gene sequencing.

S.No.	Strain	16S rRNA gene (nucleotides)	Accession no.	Species identified	Similarity index
1	NCCP-549	1419	AB719399	*Chryseobacterium gleum*	98
2	NCCP-550	1438	AB719400	*Acinetobacterium pitti*	99,5
3	NCCP-551	1152	AB719401	*Acinetobacterium pitti*	100
4	NCCP-552	1171	AB719402	*Bacillus tequilensis*	99,9

Table 3. Elemental composition (in % for N and ppm for other elements) and pH of *Peganum* smoke solutions, derived from aerial parts of plant.

Ph	N	Ca	Mg	K	Na	Fe	Mn	Cu
7.30	0.690	4.90	0.790	0.50	0.70	0.092	0.023	0.037

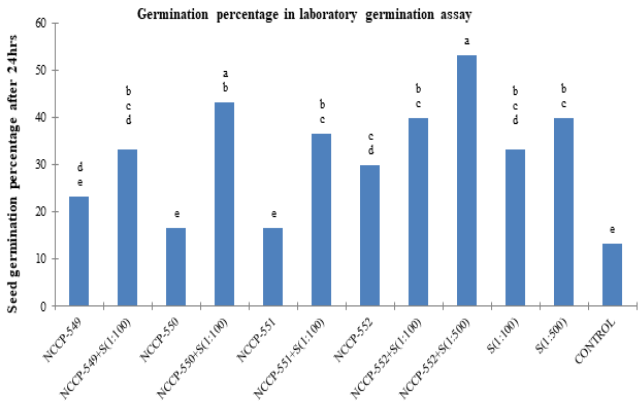

Figure 1. Influence of bacterial strains alone or along with aqueous smoke extracted from plant on germination of wheat seeds in laboratory conditions. Each bar represents mean of three replications. Different small letters on bars indicated Bars with statistically significant difference according to Fisher's LSD test at 95% confidence level. The data were analyzed for germination percentages after 24 h.

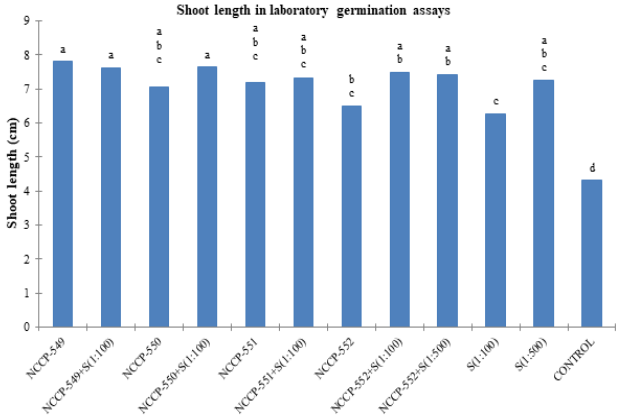

Figure 2. Influence of bacterial strains alone or along with aqueous smoke extracted from plant on shoot length of wheat in laboratory conditions. Each bar represents mean of three replications. Different small letters on bars indicated Bars with statistically significant difference according to Fisher's LSD test at 95% confidence level. The data were analyzed for germination percentages after 24 h.

The smoke solution also significantly increased the root and shoot length as compared to the control, however, the increase in this case was generally significantly smaller than that by bacterial application. When the bacteria were applied along with the smoke solution the increase in shoot and root was significant as compared to control, however, it was never more than the increase observed when the PGPB were applied alone (Figure 2 & 3). The NCCP-549 alone produced the longest roots in the present study (Figure 4).

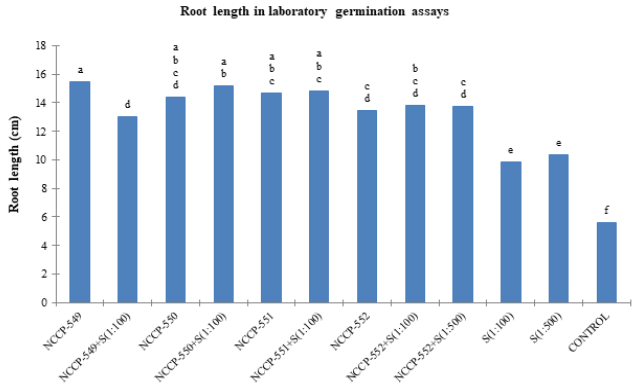

Figure 3. Influence of bacterial strains alone or along with aqueous smoke extracted from plant on root length of wheat in laboratory conditions. Each bar represents mean of three replications. Different small letters on bars indicated Bars with statistically significant difference according to Fisher's LSD test at 95% confidence level. The data were analyzed for germination percentages after 24 h.

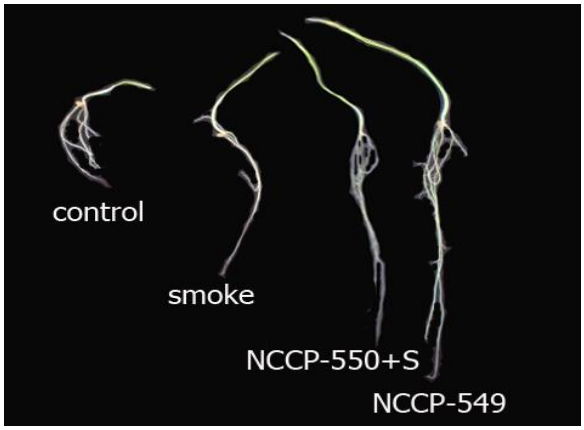

Figure 4. Root/shoot growth promotion in wheat by inoculation of bacterial strains alone or along with aqueous smoke solution in wheat under *in vitro* conditions.

Effect of inoculation and smoke treatments on plant growth parameters in greenhouse pot assays: The treatments significantly influenced the different parameters as compared to controls except the number of leaves (Table 4). The lowest fresh and dry shoot weights (1.13 and 0.26 g, respectively) were observed in control plants. The shoot weight was significantly increased by application of the rhizosphere bacteria (up to 90%) and smoke extract (up to 60%) in comparison to the non-treated control. The chlorophyll a and b contents were the lowest in control plants (5.93 and 6.76 µg/g,respectively).The chlorophyll a and b contents were the highest upon inoculation with NCCP-549 alone (7.46 and 11.01 µg/g, respectively)or along with the smoke extract (7.87

Table 4. Effect of bacteria (NCCP-549, 550, 551, 552) alone or along with aqueous smoke extracted from plant (concentrated (S (1:100)) or diluted (S (1:500))) on different parameters of wheat in greenhouse.

Treatments	Shoot weight (g)		No. of leaves	Cell membrane stability(%)	Chlorophyll Contents (µg/g)		Carotenoid contents (µg/g)	Nitrogen contents (g/g)	Protein contents (g/g)
	Fresh	Dry			C_a	C_b			
Control	1.13c	0.260e	16.66a	17.47a	5.93g	6.76d	615.13f	2.01f	12.54f
NCCP-549	2.15a	0.539a	20.66a	8.49b-f	7.46abc	11.01a	919.16a	2.70e	16.91e
NCCP-550	1.74abc	0.370b-e	18.00a	9.56bc	6.95cde	8.54bc	728.99cd	3.50bc	21.88bc
NCCP-551	1.84ab	0.389b-e	18.66a	9.16bcd	7.58ab	10.48a	845.12b	3.36c	21.00c
NCCP-552	1.71abc	0.350cde	17.66a	9.61bc	7.10bcd	9.33b	850.42b	3.54bc	22.17bc
S (1:100)	1.35bc	0.290de	17.33a	9.71bc	4.75h	7.57cd	686.40de	2.94de	18.37de
S (1:500)	1.85ab	0.417a-d	19.33a	10.29b	6.76def	9.10b	828.51b	2.94de	18.38de
NCCP-549+S (1:100)	2.10a	0.489abc	21.00a	8.96b-e	7.87a	10.71a	930.15a	3.26cd	20.42cd
NCCP-550+S (1:100)	2.19a	0.509ab	20.33a	7.27def	7.35abc	9.13b	925.13a	4.20a	26.25a
NCCP-551+S(1:100)	2.04a	0.417a-d	19.66a	6.92ef	6.66def	8.97b	948.20a	4.15a	25.96a
NCCP-552+S (1:100)	2.09a	0.462abc	20.33a	6.53f	6.30fg	7.38d	732.04c	4.20a	26.25a
NCCP-552+S (1:500)	2.06a	0.450abc	20.00a	7.98c-f	6.43efg	7.69c	680.20e	3.78b	23.63b

Note: Each value in the table indicates average of three replicates. Different small letters indicated statistically significant difference according to Fisher's LSD test at 95% confidence level. Analysis was performed separately for each parameter.

and 10.71 µg/g, respectively). The inoculation with NCCP-549 alone or along with smoke, NCCP-550 plus smoke and NCCP-551 plus smoke increased the carotenoid contents by 50%. The lowest nitrogen and protein contents (2 and 12.54 g/g, respectively) were observed in the control plants. The nitrogen and protein contents generally increased significantly when treated with the bacteria and smoke solution as compared to control or their application alone. Significant effects were also found on the cell membrane stability reducing up to 65% in comparison with controls (Table 4).

DISCUSSION

Wheat is an important staple crop in the South Asian countries where the population pressure and density are high with limited agricultural resources. For this purpose, high yielding cultivars are used along with chemical inputs to increase the yield. The present study was based on the idea of using PGPB and smoke solution together to improve growth of wheat without environmental deterioration. For this purpose, plant derived smoke solution was prepared from *P. harmala* has been reported elsewhere (Dixon *et al.*, 1995). Four bacterial strains (NCCP-549, 550, 551 and 552) were isolated from the rhizosphere of *I. aquatica* treated with smoke solution derived from *P. harmala* and were identified using the phenotypic and molecular traits. The strain NCCP-549 was identified as *Chryseobacterium*spp., NCCP-550 and 551 as *Acinetobacter* spp. And NCCP-552 as *Bacillus* spp.The 16S rRNA gene sequences of the strains were submitted in the GenBank under accession numbers as AB719399 (NCCP-549), AB719400 (NCCP-550), AB719401 (NCCP-551) and AB719402 (NCCP-552).

Bacillus spp. Have abundantly been found in the rhizosphere, and their PGPR activities and the mechanisms been extensively reported in the literature (Govindasamy*et al.* 2010). Similarly, the efficacy of *Chryseobacterium*spp. And *Acinetobacter* spp in enhancing plant growth has been reported elsewhere (Egamberdiyeva, 2005). Moreover, the smoke solution derived from *P. harmala* was previously reported to contain 35 different chemical compounds and had antibacterial activity (Shahverdi *et al.*, 2005). The bacterial strains under investigation here were found resistant to the smoke supplementation in the culture medium and, therefore, suitable for application in combination to improve the wheat growth.

The idea behind application of smoke solution and the PGPB was to accumulate the positive impact of both on the crop under investigation. We reported significantly higher seed germination rates upon treatment with smoke solution either alone or in combination with bacteria. It shows the smoke solution contained some hormone like compounds that could improve the seed germination in the present study. Daws*et al.* (2007) reported that the plant derived smoke contained the plant growth regulator like compounds which had plant growth promotory effects that might be leading to enhanced germination rates (Jamil *et al.*, 2014).

There are several reports on significance of PGPR and aqueous smoke extract derived from plants (van Staden *et al.*, 2000; Saharan and Nehra, 2011; Mia *et al.*, 2012). The different plant growth traits considered in the present work improved upon inoculation with the growth promoting bacteria alone or along with the smoke extract (Table 4). Pandey *et al.* (1998) reported the increase in fresh and dry weights of shoot, and yield of maize upon seed inoculation with bacteria. Probably secretion of growth regulating substances (auxins, cytokinins and gibberellins) by bacteria and presence of such substances in smoke solution (Daws *et al.*, 2007) seemed to be responsible for the positive effects on plant growth as observed in the present study. The smoke compounds like butenolide was reported as having gibberellins like function (Van Staden *et al.*, 2004; Malabadi

and Kumar, 2006). The most significant increase in the different growth parameters was observed in this study when the seeds were inoculated with NCCP-550 (*Acinetobacter* spp.) along with the treatment with smoke solution. Several Acinetobacter strains have been reported as plant growth promoting in addition to their ability to solubilize phosphate in soil (Bharwad and Rajkumar, 2020). The detailed review of the possible mechanisms of plant growth promotion by different microbial strains has recently been reported by Zope *et al*. (2019).

The significant increase in total chlorophyll and carotenoid contents in the present study might be due to increased N contents, stomatal conductance and metabolism (Hayman, 1983). The inoculation with *Azotobacter*(Al-Garni, 2006) or using aqueous smoke extract also significantly increased chlorophyll contents elsewhere (Jamil *et al.*, 2014). The smoke solution is also enriched with the plant nutrients that may directly impact the plant growth. Elemental analysis of smoke solution showed the presence of essential elements that may be needed not only for plant growth but also for bacteria. For instance, bacteria needed trace amounts of elements or minerals like sodium, potassium, calcium and magnesium as well as, in smaller amounts, iron, copper, manganese, zinc, cobalt and molybdenum (Banwart, 1989) and some of these elements were detected in smoke solution in the present study.

***Conclusions*:** Four bacterial strains NCCP-549, 550, 551 and 552 were isolated and identified from the plant rhizosphere treated with the aqueous plant derived smoke solution. The bacterial strains when applied alone or in combination with smoke solution enhanced the plant growth parameters of wheat. The strain NCCP-550 identified as Acintobacter spp. was found to be the most effective plant growth promoting strain when applied along with the smoke solution.

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