

CHARACTERIZATION OF CULTURE FILTRATES OF *PSEUDOMONAS SYRINGAE* PV. *SESAMI* AND *XANTHOMONAS CAMPESTRIS* PV. *SESAMI* ISOLATES ASSOCIATED WITH SESAME BACTERIAL BLIGHT

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Abstract

Different bioassays were used to detect secondary metabolites produced by *Pseudomonas syringae* pv. *sesami* (*Psse*) and *Xanthomonas campestris* pv. *sesami* (*Xcs*) virulent isolates. The bioassays were antibacterial activity, phytotoxic activity, potato tuber outgrowth and seedling assay that included qualitative, semi quantitative and quantitative. In qualitative assay, phytotoxic activity of cell free culture filtrates of *Psse*-1, *Psse*-2 and IBD-1 of *Xcs* isolates were applied on non host plant brinjal and host sesame leaves and symptoms were observed. *Psse*-2 isolate elicited water soaking and chlorosis symptoms on both tested plants as produced by pathogen, while *Psse*-1 showed only water soaking and necrosis symptoms. *Psse*-2 isolate only induced hypertrophy outgrowth in potato tuber discs, neither *Psse*-1 nor IBD-1 isolate induces this outgrowth on potato tuber discs. Antibacterial activity was also checked against three pathogenic bacteria such as *Salmonella* sp., *Pseudomonas* sp., and an unknown bacterial pathogen. Results showed that *Psse*-1 and *Xcs* isolate showed inhibition zones against only unknown bacterial pathogen but *Psse*-2 isolate did not exhibit any such zones against the tested bacterial pathogens. Moreover, biological effects of different concentrations of culture filtrates of *Psse* and *Xcs* isolates on sesame susceptible and resistant seedlings showed that all tested culture filtrates illustrated sesame root and shoot inhibition, while the inhibition recorded was more against *Psse*-2 isolate culture filtrate than others. *Xcs* and *Psse*-1 showed less inhibition and effective at 70 and 100% concentrations. Over all inhibition was less in tolerant than susceptible genotypes. Present results showed that *Psse* isolates produced two different classes of toxins, chlorosis as well as necrosis. Chlorosis inducing toxins did not show antibacterial activity but could be detected in potato tuber discs bioassay. On the other hand, necrosis inducing toxin showed antibacterial activity against unknown bacterial pathogen. Seedling bioassay also shown that chlorosis inducing toxin was more effective in inhibition of seedlings than necrosis production toxin.

Introduction

Sesame (*Sesamum indicum* L.) is one of the important conventional oil seed crops of Pakistan. Sesame seed contains 50-60 percent oil and 40 percent protein (Brar & Auja, 1979). Pakistan is facing a chronic shortage in edible oil and the situation is getting serious with alarming expansion of the population. The country's indigenous production is below its consumption level and there exists a wide gap between production and consumption. Presently, oil seed requirements of Pakistan fulfill only 30 percent of the need, and the rest is met from other crops. Different diseases are responsible to damage to the sesame crop that every now and then results in negligible or 100 percent loss in out put. Sesame crop is subjected to various abiotic and biotic stresses in all stages of growth under natural environments that interfere with sesame production. Among biotic factors air pollution showed strong effects on sesame yield (Wahid *et al.*, 2012). Two prominent bacterial pathogens associated with sesame, as bacterial blight caused by *Xanthomonas campestris* pv. *sesami* (*Xcs*) and bacterial leaf spot caused by *Pseudomonas syringae* pv. *sesami* (*Psse*) are responsible for sesame production constraints during monsoon season (Akhtar, 1986; Mirza & Akhtar, 1987; Naqvi *et al.*, 2012). Highest leaf infection occurred in plants inoculated with both the pathogens together as compared to individual inoculations (Bashir *et al.*, 2007). Genetic diversity among 20 sesame accessions was examined at DNA level by means of random amplified polymorphic DNA (RAPD) analysis (Akbar *et al.*, 2011). *P. syringae* is subdivided into 57 pathovars on the basis of

distinctive pathogenicity to one or more plant hosts (Young *et al.*, 1991 and Gardan *et al.*, 1995). It produces mainly four toxins: coronatine, tabtoxin, phaseolotoxin and lipodepsinonapeptides (syringomycin and syringopeptin) (Bender, 1998, 1999). All contribute to chlorotic or necrotic symptoms, further coronatine and phaseolotoxin has also been contributed in bacterial multiplication and spread in plants. Coronatine (COR) is produced by many pathovars of *P. syringae* and exhibits non host specificity (Cintas *et al.*, 2002). Toxinogenic strains maximized pathogen growth and increased lesion length in tomato than nontoxigenic strains of COR (Brooks *et al.*, 2004). Upon treatment of leaf tissues with COR or inoculation with the *P. syringae* strains that produced COR, chlorotic symptoms induced (Gnanamanickam *et al.*, 1982). It was considered that this chlorotic symptoms were might be due degradation of chlorophylls a and b in plants (Bender, 1999). The enzyme chlorophyllase is expressed by COR, that degraded chlorophyll pathway (Benedetti & Arruda, 2002). Many *P. syringae* strains and pathovars and some other species of *Pseudomonas*, produced *In vitro* phytotoxic and antibiotic active lipodepsipeptides (LDPs) as secondary metabolite (Ballio *et al.*, 1991; Sorensen *et al.*, 1996 and Dalla Serra *et al.*, 1999). They are thought to be major virulence factors for plant pathogenic Pseudomonads (Gross *et al.*, 1997).

Some phytotoxins have antimicrobial ability and can be easily detected using sensitive fungi or bacteria in bioassay. Growth Inhibition of *Escherichia coli* K-12 growth by phaseolotoxin is one assay for this toxin detection at picogram level (Staskawicz & Panopoulos,

1979), and tabtoxin has been detected in bioassays using toxin sensitive bacteria or fungi (Gasson, 1980). However, COR does not have antimicrobial properties, it can be detected by its ability to produce chlorotic symptoms in a variety of plants; but this assay is qualitative. A quantitative bioassay is suitable for its detection. Volksch *et al.*, (1989) have described a semi quantitative bioassay for detection of COR, where bioassay of hypertrophic outgrowth in potato tubers is used for its detection. The assay is sensitive but variation depends on potato cultivar and tuber age utilized. The most frequently used bioassay is the inhibition of root growth for a given toxin (Rasmussen & Scheffer, 1988a).

Materials and Methods

Bacterial isolates and culture conditions: In the present study isolate *Psse*-1 (Moderately virulent), *Psse*-2 (Strongly virulent) and IBD-1 (Strongly virulent) of *Xcs* were used that were previously maintained on Nutrient Glycerol Agar (NGA) at 4°C (Bashir, 2007 and Ahmad, 2004). The purity of *Psse* isolates were confirmed on nutrient broth sucrose agar (NSA) dishes (Lelliot & Stead,

1987) by incubating at 25°C for 48 h. *Xcs* isolate was streaked on Yeast Dextrose Calcium Carbonate Agar (YDCA) medium for confirmation of yellow colonies. Pure culture were kept in glass culture tubes containing 10 ml of NGA (Lelliot & Stead, 1987) grown at 25°C for 48 h and again maintained at 4°C.

Bioassays and Detection

Stationary culture of bacterial isolates: Initially, *Psse* and *Xcs* isolates were tested in bioassay for their antibacterial activity through growth test against available bacteria such as *Salmonella* sp., *Pseudomonas* sp., and *Bacillus megaterium* ITM 1458 provided by the Department of Plant Pathology University of the Punjab, Lahore, Pakistan. *B. megaterium* ITM 100 was reported to be sensitive to SPs (Lavermicocca *et al.*, 1997). Each isolate was grown on following substrates. Potato Dextrose Agar (PDA), supplemented with casamino acid (PDCA), King's B medium (Kings *et al.*, 1954) and Improved Minimal Medium Agar (IMMA), a modified of IMM (Surico *et al.*, 1988). The composition of each media was annexed in Table 1.

Table 1. Composition of different media (The composition is for 1 L).

Medium	Ingredients	Quantity (g)
Yeast Sucrose Peptone Agar (YSPA)	Yeast extract	5
	Peptone	10
	Sucrose	20
King's B medium (KB)	Proteose peptone	20
	Di-potassium hydrogen phosphate	10
	Magnesium sulphate	1.50
	Glycerol	15
Improved minimal medium (IMM)	L-histidine-HCL	4
	Mannitol	10
	Mg SO ₄	0.2
	Ca Cl ₂	0.1
	FeSO ₄	0.02
	KH ₂ PO ₄	0.8

Antibacterial activity: An agar spot test was used to detect antibacterial activity. Bacteria to be tested were grown on NA for 48 h at 25°C. Twenty microliter aliquots of the *Psse* and *Xcs* isolates from 48 h old culture (approximately 1x10⁸ CFU/ml) was spotted on above mentioned media. Each dish was spotted in three equidistance places corresponding to the three points of a triangle. The plates were incubated for 4 days at 27°C. They were then over sprayed with a cell suspension of each of the three indicator bacteria prepared by dissolving a loopful of 48 h old culture streaked on NA in 10 ml of SDW. After spraying, dishes were left to dry in a sterile laminar flow hood and then incubated at 27°C for 2-4 days. The presence of a clear zone around *Psse* and *Xcs* colonies indicated the inhibitory effect. The experiment was repeated twice (Cirviller *et al.*, 2005).

Preparation of cell free culture filtrates: *Psse* and *Xcs* isolates were grown overnight in NGA and 1 ml of bacterial suspension (OD 600= 0.3) was added in 1 L

Roux flasks containing 100 ml of modified IMM liquid medium as well as in 250 ml conical flasks containing potato dextrose broth plus Casamino acid (PDBCA) with pH 5.5 as stationary cultures at 25°C in darkness (Lops, 2001) for 7-10 days. After incubation in still culture and centrifugation at 9000 x g for 20 min, the supernatant was passed through a 0.22 µm syringe filter for cell free culture filtrates. For each isolate 5 flasks were inoculated each with 1 ml aliquot of suspension prepared in SDW with a 24 old bacterial culture grown on NGA. Moreover, 50 µl of an overnight *Psse*-2 culture was added to 1 ml of Hoitink and Sinden medium (Hoitink & Sinden, 1970) amended with sucrose (HSS). The pH was adjusted to 6.5 with 10 M NaOH at the time of autoclaving. Sucrose and FeCl₃ were autoclaved separately. After sterilization, medium was incubated on a rotary shaker at 20°C for 7 days by shaking at 250 rpm. After incubation, centrifuged 1 ml of this bacterial suspension at 2,000 x g was kept for 10 minute at room temperature.

Phytotoxicity assay: The phytotoxic activity of cell free culture filtrates of the *Psse* and *Xcs* isolates was evaluated on Brinjal (*Solanum melongena*) and sesame leaves. Cell free culture filtrates, prepared as described above, were inoculated into the lamina of fully expanded leaves of above mentioned plants with a hypodermic syringe. Control inoculated only with SDW. Plants were then maintained in growth chamber. The appearance of water soaked, chlorotic as well as necrotic symptoms were recorded after 48 h as positive for the production of toxin (Cirviller *et al.*, 2005).

Test for hypertrophy in potato tuber: Production of chlorosis producing toxin was evaluated by a semi quantitative potato disc bioassay (Volkch *et al.*, 1989). Twenty microliter cell free culture filtrate as described above was spotted onto the potato discs. The presence of chlorosis producing phytotoxin was characterized by a hypertrophic response (enlargement of tissue) on the potato discs. Bacteria have also been assayed directly for hypertrophic outgrowth by placing a loopful of inoculum on the surface of the potato tuber discs. Tissue was assessed for hypertrophic outgrowth 5 days after inoculation.

Seedlings assay: Seedlings of sesame were grown on moist filter paper for 3 days in darkness at 30 °C. Seedlings with uniform hypocotyl and root lengths were transferred to filter paper saturated with the respective concentration. These seedlings were then treated with 2 ml of different concentrations of culture filtrates of *Psse* and *Xcs* isolates prepared in SDW (0, 30, 50 70 and 100%). Seedlings were further incubated in light for 3 days at 30°C. Photographs were taken 3 days after treatment and the nearest 0.1 cm on scale was selected for the measurement of the root and shoot length. Ten independent seedlings were selected for mean from three independent experiments for each treatment.

Results and Discussion

Antibacterial activity: Antibacterial activity of *Psse* and *Xcs* isolates were tested against *B. megaterium*, *Salmonella* sp. and *Pseudomonas* sp., on 4 different media such as PDA, PDCA, KB and IMMA. None of the pathogen showed antibacterial activity against the tested pathogen and media. However, *Psse-1* isolate showed antibacterial activity against an unknown bacterial pathogen by PDA plate assay. This unknown bacterium was introduced during work on isolation of *Psse* and *Xcs* from disease discs plated on NGA in laboratory and inhibitory zones were observed. To check which bacterium displayed this inhibitory activity, another test was made wherein first *Psse* and *Xcs* isolates were agar spotted on PDA as described in materials and methods. These plates were over sprayed with 24 h old suspension of unknown bacterium. On the other hand, unknown bacterium was spotted as described above and over sprayed with *Psse* and *Xcs* 24 h old bacterial suspensions.

On confirmation, it was observed that *Psse* and *Xcs* showed inhibitory zones against unknown bacterium. It was also observed that *Psse* and *Xcs* pathogens did not produce LPDs in bioassay with *B. megaterium*, in antibacterial tests as LPDs (Syringopeptins) act as antibiotic agents and showed inhibitory zones against *B. megaterium* (Laverimicocca *et al.*, 1997). Isolate *Psse-2* did not show inhibitory activity against the tested bacterial pathogens. The ability of chlorotic producing isolates to induce potato outgrowth as well as without any antibacterial properties indicated that this isolate of *P. syringae* produce more than one toxin with different biological activities.

Phytotoxic activity: Culture filtrates of *Psse* as well as *Xcs* isolate were phytotoxic to brinjal and sesame. On brinjal *Psse-2* showed water soaking, chlorotic symptoms similar to those caused by the pathogen (Fig. 1A), while *Psse-1* did not show any chlorosis but showed water soaking and necrosis symptoms (Fig. 1B) with water soaking were more pronounced on lower side (Fig. 1C). *Xcs* showed typical necrosis on sesame leaves as those caused by the whole cells (Fig. 1D). Moreover, it was also noticed that *Psse-2* showed chlorosis and traveled along the veins. The reason behind this scenario was not cleared but might be due to the infection of pathogen in vascular tissues, either xylem or phloem. No phytotoxic symptoms were developed on plants inoculated with SDW. The development of symptoms on non host plants using culture filtrates also indicated that the toxins produced by these pathogens were non host selective. This phytotoxic assay is qualitative rather a semi quantitative and qualitative assays were most suitable such as potato outgrowth, antibacterial test as well as seedlings assays for the detection of bioactive compounds. However, this assay was also suitable for preliminary detection of any bioactive compound, because the culture filtrate showed similar lesions as produced by the pathogen under natural conditions. Therefore, one of the important aspects to work on these toxins was that toxins could help in screening of varieties within short duration of time compared with old screening methods which were time consuming and labour intensive. In the near future, toxins can be used in disease management strategies.

Effects of chlorosis (*Psse-2*) and necrotic (*Psse-1*) inducing isolates of *Psse* and *Xcs* on hypertrophy response of potato tuber discs: Isolates of *Psse* as well as *Xcs* isolate were tested for their ability to cause hypertrophy of potato tuber discs. Outgrowth developed on tuber slices inoculated with *Psse-2* isolate; however, *Psse-1* and *Xcs* isolates remained flat and smooth (Fig. 2). Presence of hypertrophic outgrowth by *Psse-2* strongly indicated the involvement of COR like phytotoxin. The absence of any hypertrophic outgrowth by *Psse-1* and *Xcs* suggested that these isolates did not produce chlorosis inducing phytotoxin COR. In the present study only *Psse-2* isolate of *Psse* induced hypertrophic outgrowth,

but *Xcs* and necrotic (*Psse-1*) did not show any cell enlargement. So present results were consistent to that of Sakai *et al.*, (1979) and it was concluded that this outgrowth was due to COR or related toxin. Phytotoxin COR is not antimicrobial, and the hypertrophic outgrowth in potato tuber is best assay for its detection (Sakai *et al.*, 1979). COR is a chlorosis eliciting toxin produced by many pathovars *P. syringae* (Ichihara *et al.*, 1977; Mitchell & Young, 1978 and Mitchell *et al.*, 1983). COR production appeared to be important for infection because chlorosis defective strains did not produce this toxin and reduced virulence (Bender *et al.*, 1987). COR had adverse effects on plants such as promotion of senescence in tobacco (Kenyon & Turner, 1990), inhibition of wheat root growth (Sakai, 1980), and promotion of cell enlargement (Sakai *et al.*, 1979b) in potato tuber tissues. Development of outgrowth in potato tuber indicated that COR might mimic the action of one of the phytohormones such as auxins, cytokinins, gibberellins, abscisic acid or ethylene. It is generally suggested that auxin promotes water uptake by discs of potato tuber tissue (Van Overbreek, 1944). On the other hand several researchers have studied the

similarity between COR and methyl jasmonate (MeJA), a plant growth regulator, on the basis of similarity in structure and functions (Wasternack & Parthier, 1997). For example, oat leaves senescence (Ueda & Kato, 1980), inhibits arabidopsis root growth (Staswick *et al.*, 1992) and potato tissue enlargement (Koda *et al.*, 1991) were due to MeJA. In present investigation, *Psse-2* isolate induced hypertrophic outgrowth in potato discs but did not induce by chl- and *Xcs* isolates. It is concluded that chlorosis producing symptoms was due to the action of chlorosis inducing toxin COR, and *Psse-2* isolate produced phytotoxin COR as conformation was done in potato tuber assay. So present result supported the work of Sakai *et al.*, (1979 and 1979b) and Volksch *et al.*, (1989) who reported the presence of COR was characterized by enlargement of potato tissues using pure culture or culture filtrate of *Psse*. It is well studied that plant pathogenic microorganisms produce physiologically active compounds that can regulate the growth of plants. COR is an extracellular toxin produced by many pathovars of *P. syringae* and have the ability to deform the tissue and elongate the cells by water uptaking.



Fig. 1. Water soaking and chlorosis symptoms on brinjal due to culture filtrate of *Psse-2* isolate **A**, necrotic due to necrotic (*Psse-1*) producing isolates on same plant in **B** and water soaking in **C**. *Xcs* isolate induced blight like necrosis on sesame in **D**.

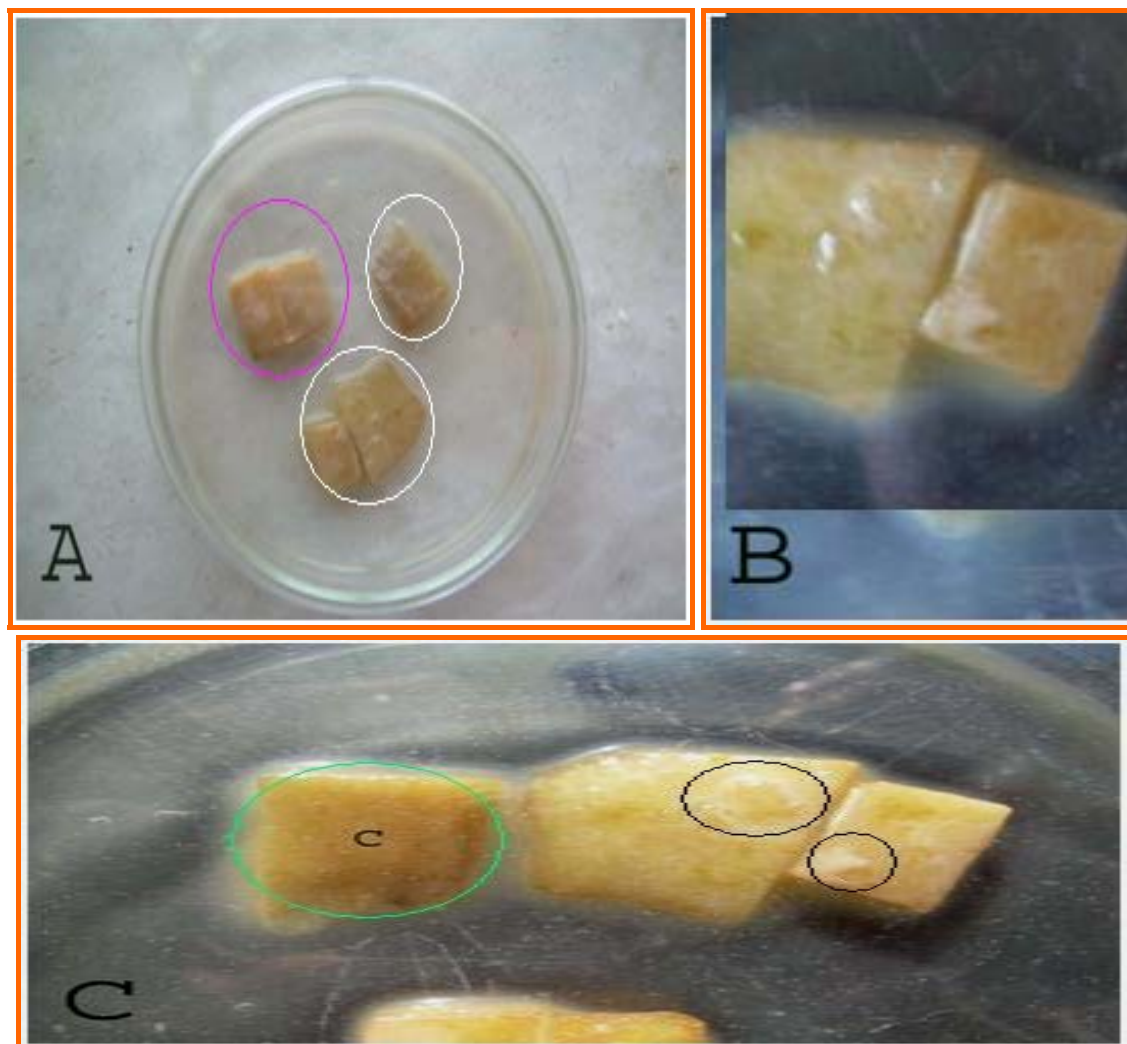


Fig. 2. Cell enlargements due to *Psse-2* isolate encircled in white, necrotic (*Psse-1*) isolate did not show any enlargement encircled in pink in A. Close images of cell enlargement due to *Psse-2* in B and C.

Seedlings assay: In the present study sesame seedling were assay to study the biological activity of culture filtrates. Culture filtrates were prepared on different media as described in materials and methods. Different concentration (0, 30, 50, 70 and 100%) of culture filtrates (approximately 2 ml) of *Psse* and *Xcs* isolates were applied 2 times within 4 days. Results showed that culture filtrates of *Psse-2* isolate inhibited both root and shoot length of susceptible (GP-9) and tolerant (Gp-34) genotypes. On increasing concentration, root length also decreased as compared to control (0%). Similarly, susceptible genotypes showed less inhibition than tolerant genotypes except control (Fig. 3A). Both susceptible and tolerant showed root length approx. 0.193 cm on concentration 0%, 0.181 and 0.105 cm on 30%, 0.136 and 0.082 cm on 50%, 0.094 and 0.062 cm on 70% and 0.063 and 0.049 cm by using pure culture filtrate (Fig. 3A). However, effect of culture filtrate on shoot length was not as strong as in susceptible genotypes, concentration 0 and 30 % showed similar shoot length that was approx. 0.199 cm. After that on

increasing concentration shoot showed reduction in length where it was measured approx. 0.169, 0.08 and 0.048 cm by using concentration 50, 70 and 100% (Fig. 3B). Similarly, tolerant genotypes also showed profound changes on increasing concentration but showed lower shoot length as compared to control (Fig. 3B). Culture filtrates of *Psse-1* and *Xcs* isolate did not show strong inhibition that were able to measure except at 70 and 100% (Fig. 3C). These results are consistent with previous investigations made by different workers. In this regards, Sakai (1980) compared the physiological activities of COR with those of indol-3-acetic acid (IAA) in root elongation of wheat and hypocotyls elongation in mungs bean. In root elongation of wheat, both COR and IAA showed similar activity, but hypocotyl segments length was slightly affected by COR and markedly stimulated by IAA. He deduced that COR has the typical activity of auxin in inhibit of root elongation, whereas it lacked this ability in mung bean hypocotyl elongation. Other studies shown that root growth directly affected by

COR, CFA, and MeJA (Staswick *et al.*, 1992; Feys *et al.*, 1994 and Tang *et al.*, 1996). Another study was done in this regard by Uppalapati *et al.*, (2005) for investigation of the biological effects of COR and its components CFA and CMA as well as one of the growth regulator, MeJA on tomato seedlings using different concentration such as COR (0.002, 0.02, 0.2, 2 or 20 nmol), CFA with same concentration as COR, CMA (0.02, 0.2, 2, 20, 200 or 2000 nmol), MeJA (20, 200 or 2000 nmol) and ACC with same concentration as MeJA with addition of 0 nmol. Results showed that

tomato root growth was inhibited by COR even at low concentrations. Root growth was also inhibited by CFA at higher concentration than that observed in COR. Kenyon & Turner (1992) shown that there is no distinction between COR and ethylene precursor 1-amino cyclopropane carboxylic acid (ACC) in hypertrophy of potato tubers and necrotic symptoms in tobacco leaves. The actual mechanism of these responses was not well understood, but it was thought that this biological effect was might be due to phytohormones like substance also produced by *Psse*.

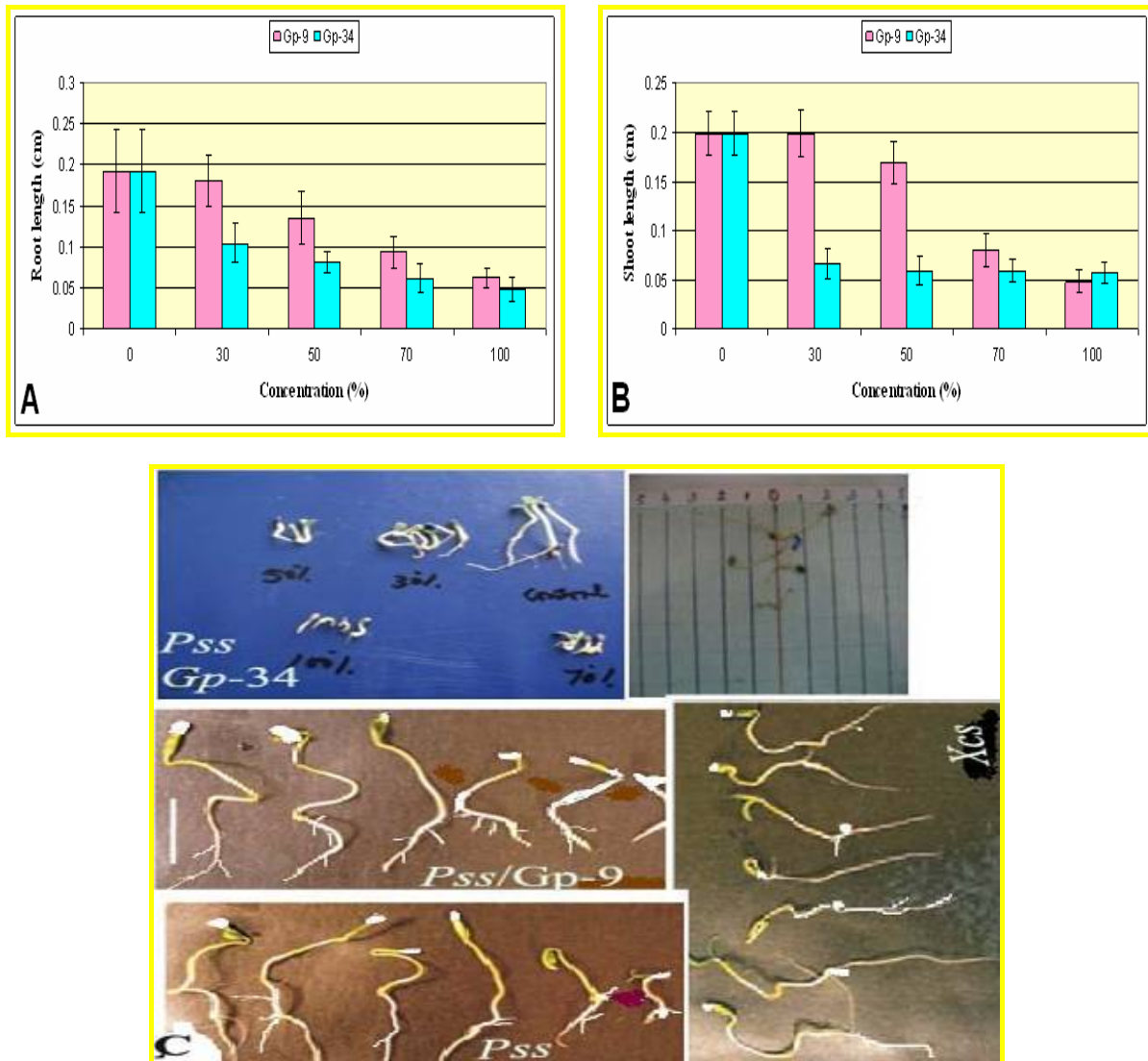


Fig. 3. Effects of different concentrations of culture filtrate of *Psse-2* isolate of *Psse* on root and shoot length. **A** and **B** showed decreased root and shoot length on increasing conc. **C**, showed effects by *Psse-1* and *Xcs* on root and shoot.

Conclusions

It was concluded that *Psse* isolates produced two types of active compounds (toxins), some showed antibacterial activity while others did not. Moreover, *Psse-2* isolate of *Psse* produced chlorosis producing toxin like COR. Further confirmation was made in bioassays of potato tuber

outgrowth and inhibition of seedlings. Hypertrophic outgrowth in the present study confirmed that *Psse-2* produced coronatine like toxin such as those produced by *P. syringae* pv. *tomato*. Further seedlings assay supported these results. The hypertrophic outgrowth and seedlings assay demonstrated the facts that toxin functions like one of the plant growth substances or plant growth regulator

MEJA. Further chemistry is required for the confirmation at this stage. The most efficient bacterial ethylene producers are plant pathogens belonging to certain pathovars of *P. syringae*, as *P. syringae* pvs. *glycinea* and *Phaseolicola* and might be *P. syringae* pv. *sesami* as shown in present investigations of potato tuber outgrowth. The findings of the present results can open new vistas in future on this important bacterial pathogen because Coronatine is of interest to both plant biologists and plant pathologists, as it appears to function as a molecular mimic of two different endogenous hormones, JA and ethylene both of which can play important roles in plant defence. On the other hand, ethylene also functions as a gaseous hormone. Based on these investigations, *Psse* can also be used for numerous purposes, and so future work on this important bacterial pathogen can help in solving many problems in both Biology and Plant pathology field.

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