



Protection of Eggplant Seed Germination from the Inhibitory Effect of *o*-Cresol by Bioaugmentation of Soil with *Pseudomonas monteilii* Strain

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAMK designed the experiments and wrote the protocol. Authors SVM and AAMK wrote the first draft of the manuscript. Authors TCS and MA executed the experimental work. Author TCS managed the literature search. All authors read and approved the final manuscript.

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ABSTRACT

Bulk production and widespread end use of cresol isomers in various industrial processes result in their ubiquitous presence in the environment. Cresols are highly toxic to both fauna and flora and are included in the list of priority pollutants. This study presents the effect of *o*-cresol on germination of 10 different vegetable crop seeds as tested by the standard Filter Paper Method. The seeds of eggplant and long-podded cowpea were found to be highly sensitive. The most sensitive eggplant seeds were subjected to further studies in soil. Germination percentage and the seedling vigor were drastically reduced in the presence of *o*-cresol even at a concentration as low as 50 mg kg⁻¹ soil. A number of abnormalities in the seedlings such as stunted root and shoot growth, non-emergence of primary leaves, and negative geotropic growth were observed. Standard 2, 3, 5-tetrazoliumtrichloride test showed marked reduction in the viability of eggplant seeds proportionate to the concentration of *o*-cresol (0 through 200 mg L⁻¹) they were exposed to, which reached zero at 175 mg *o*-cresol L⁻¹, indicating the inhibition of the respiratory enzymes of the seeds. Contrary to earlier reports on the effect of phenolics on the hydrolytic enzymes of

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germinating seeds, in the present case an enhanced activity of amylase was observed in the presence of *o*-cresol (50 and 150 mg kg⁻¹ soil), whereas the protease activity was partially inhibited at higher concentration. The inhibition of seed germination by *o*-cresol was revoked by bioaugmentation of the soil with the cresol-degrading *Pseudomonas monteilii* S-CSR-0014 (2.3 x 10⁸ CFU g⁻¹ wet soil) enabling normal seed germination and seedling growth. The inoculated bacterium degraded 50 and 150 mg *o*-cresol kg⁻¹ soil efficiently, with concomitant growth. It can be concluded that by bacterial bioaugmentation of *o*-cresol-contaminated soils the inhibition of germination of crop seeds could be eliminated effectively enabling healthy seedling growth.

Keywords: Seed germination inhibition; pseudomonas; cresol-degradation; inhibition elimination.

1. INTRODUCTION

Cresol isomers viz. *ortho*-, *meta*-, and *para*-cresol (*o*-, *m*-, and *p*-cresol) are methylated phenol that are classified so based on the position of the methyl group on the benzene ring, which are used extensively in industry. *o*-Cresol is used directly as either a solvent or disinfectant and as a chemical intermediate in the synthesis of a variety of products such as deodorizing agents, antiseptics, fragrances, antioxidants, dyes and dye intermediates, resins, herbicides, pesticides, and other consumer goods and its worldwide market is expected to grow fast and will reach 150 million USD in 2024 [1].

o-Cresol and other isomers are harmful to both fauna and flora [2]. United States Environmental Protection Agency has classified cresols as stable priority chemical pollutants [3]. Cresols are also generally found in soils in low levels as they are released *via* excrement, exocellular secretions, and necromass of living and dead organisms, where they are expected to degrade rapidly [2,3]. However, they can become highly harmful to the ecosystem when they enter the environment in high concentrations as inadequately treated industrial effluents or as seepages from dumpsites and land-fillings [2,3].

Reports on phytotoxicity of cresols, particularly on inhibition of seed germination, which is the process of emergence and growth of embryo to young plants by rupture of seed coat, are very scarce, except the following: Toxicity of *o*-cresol on seven Chinese vegetables as indicated by inhibition of seed germination and root elongation has been reported [4]. It has been shown that mustard and okra seed germination was drastically affected by *o*-cresol [5]. Recently, severe inhibition of eggplant and okra seed germination by *m*-cresol has been reported [6]. However, literature is replete with information on the phytotoxic effects including seed germination inhibition of a number of other phenolic compounds. Work on allelopathy of these

compounds have been compiled and reviewed by various authors [7–9]. Auto-allelopathic effect of cinnamic acid and vanillin secreted as root exudate of eggplant that get accumulated in the rhizosphere soil at concentrations higher than 1 mmol L⁻¹ on repeated monocropping have been shown to drastically inhibit the seed germination and the root growth of the seedlings [10]. A dose-dependent improvement in growth and physiology of eggplants has also been demonstrated by amendment of eggplant monocropped soil with raw garlic stalk [11]. It has been shown in several studies that the phytotoxic effects of olive mill wastewater and dry olive residue were due to the presence of low molecular weight phenolic compounds in them and attempts to remove them by various means have also been reported [12–16].

Drastic reduction in germination and seedling vigor of chickpea, mung bean, and long-podded cowpea in the presence of phenol has been demonstrated recently [17]. Retardation of corn plant growth in the presence of phenol [18,19] and phytotoxicity of phenol and 2,4,6-trichlorophenol on local agricultural plant species in China [20] has been reported. Technical hexachlorocyclohexane (tech-HCH) was shown to inhibit germination of radish and green gram seeds [21]. It has been reported that seedlings of peas and soybean were susceptible to the herbicide, dicamba (3,6-dichloro-2-methoxybenzoic acid) [22]. The inhibitory effect of 2,4,5-T on various crop seeds has been reported, the effect being severe on eggplant and tomato seeds [23]. Severe inhibition of tomato seed germination on exposure to 3-chloro- and 4-chlorobenzoates (3-CBA and 4-CBA) has also been demonstrated [24].

As *o*-cresol is a bulk chemical used in various industrial processes there lies the possibility of its entry, in toxic levels, to cultivating fields from waste dumpsites as well as from industrial wastewaters disposed without proper treatment. In such situations it becomes imperative to

remediate the soils before their use for cultivation. Bioremediation through microbial bioaugmentation is being considered now as an effective way of removing toxic chemicals from polluted sites [25,26]. Effective methods of elimination of cresols from contaminated soils and wastewaters employing cresols-degrading microorganisms have been developed by several workers [27–30]. It has been reported that the inhibitory effect of *o*-cresol on mustard seed germination could be revoked through inoculation of the contaminated soil with the cresol-degrading strain *P. monteilii* SHY [5]. Bioaugmentation of *m*-cresol-contaminated soil with *P. monteilii* S-CSR-0014 was shown to eliminate the inhibitory effect of *m*-cresol on okra seed germination [6]. Protection of various crop seeds from inhibition by different chemicals such as phenol, tech-HCH, dicamba, 2,4,5-T and 3-CBA/4-CBA through bioaugmentation of soils with microbes capable of degrading the respective compound has also been reported [17,21–24].

We have been studying the effect of different phenolic and other aromatic compounds including phenol, *m*-cresol, 2,4,5-T and 3-CBA/4-CBA on germination of different crop seeds and how the toxic effect could be eliminated [6,17,23,24]. As a part of that we studied the effect of *o*-cresol on germination of crop seeds with particular reference to eggplant (*Solanum melongena* L). Eggplant (brinjal or aubergine) is an important commercial vegetable crop of subtropics and tropics extensively grown as an annual crop in warm areas of India, Bangladesh, Pakistan, Myanmar China, and Philippines which also is popular in Egypt, France, Italy and USA [31]. Presented here are the data on the effect of *o*-cresol on germination and seedling growth of 10 different seeds, with emphasis on eggplant and how the inhibition was eliminated by bioaugmentation of the *o*-cresol-spiked soil with a cresol-degrading bacterial strain, *Pseudomonas monteilii* S-CSR-0014, which was isolated and studied earlier in the laboratory [6,30].

2. MATERIALS AND METHODS

2.1 Soil

The red loamy type soil was collected from the campus of SAFI Institute of Advanced Study (SIAS). The soil had approximately the following composition: clay-15%, silt-40%, sand-45%, organic matter 1.0–1.5%, and a good water holding capacity.

2.2 Seeds

Seeds of spinach (*Spinacia oleracea*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*), pumpkin (*Cucurbita maxima*), okra (*Abelmoschus esculentus*), red chili (*Capsicum annum*), chickpea (*Cicer arietinum*), green gram (*Vigna radiata*) and long-podded cowpea (*Vigna unguiculata* subsp. *sesquipedalis*) used in the study were procured from the local market at Ramanattukara, Kerala. All the seeds were subjected to an initial screening by Filter Paper Method for their susceptibility to *o*-cresol. Then the most vulnerable eggplant seed was selected for detailed studies by Soil Method.

2.3 Microorganism

The cresol and phenol-degrading bacterial strain, *P. monteilii* S-CSR-0014 used in the study for bioaugmenting the soil was isolated earlier in the laboratory by enrichment of soil collected from the vicinity of a petrol bunk with cresols as sole carbon source [6,30]. The strain was Gram negative, mobile, un-capsulated, rod-shaped, aerobic, catalase and oxidase positive, non-fermentative bacterium forming tiny, circular, translucent, colonies on mineral agar plates containing 500 mg *o*-cresol L⁻¹. The strain was capable of utilizing 1000, 1200, and 1500 mg L⁻¹ of *ortho*-, *meta*-, and *para*-cresols, respectively as sole source of carbon [30].

2.4 Seed Germination Tests

Germination test of seeds exposed to *o*-cresol by filter paper method was conducted according to a slightly modified method of International Seed Testing Association (ISTA) [32] as described earlier [6,17] as follows: Two layers of filter paper discs (9 cm) were placed in each 10 cm Petri dish and moistened with solutions containing 0, 100, 200, 400, 600, 800 and 1200 mg *o*-cresol L⁻¹. In each Petri plate 25 seeds were placed and incubated in a germinator at ambient temperature (20-27°C) under 12 h cycles of light and darkness. For each concentration of the chemical, 3 replicates of 25 seeds were taken. Germination percentage (GP) and seedling vigor (SV) were evaluated after 7 days by counting the seedlings and measuring the root and shoot lengths. The SV was expressed as Vigor Index (Vi) of the seedlings which was computed using the following equation:

$$Vi = (MSL + MRL) \times \%Gr$$

Where, MSL - mean shoot length, MRL - mean root length, %Gr - germination percentage.

Germination test in soil was conducted as follows: The soil was sieved (2 mm) to remove debris and pebbles and autoclaved at a pressure of 15 psi (121 °C) for 20 min initially and then for 60 min after 2 days. Sixty g each of soil (20% moisture) was filled in alcohol-sterilized and dried plastic cups of 11 cm diameter and 4 cm depth, *o*-cresol solution was added at concentrations of 0, 25, 50, 100, 150, and 200 mg kg⁻¹ soil, and mixed thoroughly to obtain uniform distribution. The sides of the cups were pricked with a needle to enable aeration. Later, in the bioaugmentation studies 0, 50, and 150 mg *o*-cresol kg⁻¹ soil was used. Twenty-five eggplant seeds were placed in each cup at equal distance at a depth of 0.5 cm and 3 replicates were taken for each variable. The cups were incubated in a germinator as described above for filter paper method. Sterile distilled water (5 ml) was added to each cup every alternate day by sterile pipets to maintain moisture. After 7 days the GP and the Vi were evaluated as described above.

2.5 Assay of Enzymes

The protease and amylase activities in eggplant seeds sown in soil cups containing *o*-cresol as well as in control cups were estimated as follows: Duplicate samples of 10 seeds/seedlings were collected every 24 h for 7 days and were ground with acid-washed sand for 15 min in a cooled mortar maintained on an ice-bath. The extract of protease was prepared in 0.2 M phosphate buffer (pH 8.03) and of amylase in 0.2 M acetate buffer (pH 5.2) and the debris was removed by centrifugation at 10,000xg for 10 min at 4°C. The supernatants were made up to 5.0 ml.

A slightly modified method of Laskowsky [33] was followed for protease assay using bovine serum albumin (BSA) as substrate, as described earlier [6,17]. To a solution of BSA (10 mg ml⁻¹) an equal volume of enzyme extract was added and incubated at 30 °C for 30 min. The enzyme activity was expressed as OD₆₀₀ of the reactant of BSA hydrolysate with Folin-Ciocalteu reagent, which was determined using Shimadzu UV-1650 PC spectrophotometer.

Amylase activity was assayed by measuring the release of reducing sugar from gelatinized soluble starch (1.0% in 0.1 M acetate buffer, pH 5.2) according to the method of Bernfeld [34], as described earlier [6,17]. The reducing sugar released was reacted with dinitro salicylic acid

(DNS) reagent and the absorbance of the reactant solution was measured at 540 nm using Shimadzu UV-1650 PC spectrophotometer, after appropriate dilution.

2.6 Seed Viability Test

Viability of eggplant seeds exposed to *o*-cresol was tested using 2,3,5-tetrazoliumtrichloride (TTC) by the procedure of ISTA Rules 2009 [35] as previously described [6,17]. Ten seeds were soaked for 24 h in solutions of *o*-cresol of different concentrations viz. 0, 25, 50, 75, 100, 125, 150, 175, and 200 mg L⁻¹, separately. The soaked seeds were cut along the margin to expose the embryo and placed in 0.1% aqueous solution of TTC for 24 h at 37 °C in darkness. The seeds were then removed, washed with distilled water, and soaked in 10 ml of 95% ethanol until all the color was extracted. The optical density of the extracted red color was determined at 480 nm using a spectrophotometer (Shimadzu UV-1650 PC).

2.7 Bioaugmentation of Soil and Bacterial Growth Determination

2.7.1 Preparation of bacterial inoculum

Cell biomass of *P. monteilii* S-CSR-0014 was prepared by growing the culture in a fermenter (Murhopye Scientific Company, Mysore) in a mineral medium described earlier [6] containing the following composition (g L⁻¹): KH₂PO₄ - 2.72, Na₂HPO₄ - 3.52, NH₄(SO₄)₂ - 0.50, Yeast extract - 0.050, MgSO₄.7H₂O - 0.2, Ca(NO₃)₂ - 0.1 and one ml of trace mineral solution containing (g l⁻¹) FeSO₄.7H₂O - 1.0, MnSO₄.H₂O - 1.0, NaMoO₄.2H₂O - 0.25, H₃BO₃ - 0.1, CuCl₂.2H₂O - 0.25, NH₄NO₃ - 0.10, Ca(NO₃)₂.6H₂O - 0.25, NiSO₄.6H₂O - 0.19, Conc. H₂SO₄ - 5 ml. The pH of the medium was 7.2 before autoclaving. *o*-Cresol was added at 500 mg L⁻¹ level to the medium after cooling. The pre-inoculum grown for 24 h in 100 ml of the same medium in a 500 ml shake flask at 30°C was added to 2 liters of the medium taken in a 3-liter fermenter jar. The fermenter was run at 30°C with an aeration rate of 1 v⁻¹v⁻¹m⁻¹ and a propeller speed of 250 rev. min⁻¹. The bacterial cells were harvested by centrifugation (10,000xg) at 4°C for 20 min after 48 h of growth and the cells were suspended in 50 ml of sterile physiological saline.

2.7.2 Bioaugmentation of soil and determination of bacterial growth

Three ml each of the bacterial suspension was inoculated to 60 g soil (20% moisture) containing

0, 50, and 150 mg *o*-cresol kg⁻¹ soil taken in plastic cups and mixed thoroughly to ensure uniform distribution. This amounted to an inoculum size of about 2.3×10^8 colony forming units per gram wet soil containing 20% moisture (CFU g⁻¹ wet soil). Another set of cups containing similar amounts of *o*-cresol was maintained as un-inoculated control. Soil without *o*-cresol was also inoculated to serve as a bacteria-augmented control. In all cases, 3 replicates of cups were taken. Seeds of eggplant were sown immediately and on 4th and 8th day after cell bioaugmentation, separately in 3 different sets. The cups were incubated in a germinator at ambient temperature (20-27°C) and the seedlings were evaluated for GP and SV after 7 days as described above.

The survival and the growth of the inoculated bacterium was determined as follows: One g sample of the wet soil (20% moisture) was collected at 24 h intervals and suspended in 10 ml of sterile saline (0.9% NaCl) and shaken in a Vortex mixer. The bacterial suspension was appropriately diluted and 0.1 ml was spread on agar plates containing mineral media with *o*-cresol (500 mg L⁻¹) as substrate. The plates were incubated at 30°C for 24-48 h. The colonies were counted using a colony counter and the growth was expressed as CFU g⁻¹ wet soil.

2.8 Estimation of *o*-Cresol

Residual *o*-cresol in spiked and bacterially amended and unamended soils was estimated by a modified 4-aminoantipyrene colorimetric method based on Lacoste et al. [36] as described previously for *m*-cresol [6]. One g soil was collected from each cup at 24 h intervals and *o*-cresol was extracted in 5 ml distilled water by mixing gently. To 10 ml of appropriately diluted sample of the extract 0.5 ml of borate buffer, 0.1 ml of 1.5% 4-aminoantipyrene and 0.1 ml of 10% potassium ferricyanide (K₃Fe(CN)₆) solutions were added. The color developed was measured at 506 nm using Shimadzu UV-1650 PC spectrophotometer. Results were computed from a standard calibration curve prepared using varying concentrations of *o*-cresol.

2.9 Statistical Analysis of Data

Duncan's multiple range test (DMRT) was employed to determine the statistical significance ($P = .05$) of the differences among the mean values. Significant differences were indicated by different letters in the tables.

3. RESULTS AND DISCUSSION

3.1 Effect of *o*-Cresol on Seed Germination

The 10 crop seeds screened by filter paper method showed different response to *o*-cresol. Eggplant and long-podded cowpea seeds were the most sensitive among them (Fig. 1A and B). Marked reduction in GP and SV was observed in these cases even at a concentration as low as 50 mg *o*-cresol L⁻¹. Only partial germination with reduced SV was exhibited by cucumber and okra seeds up to a concentration of 200 mg *o*-cresol L⁻¹ and 800 mg *o*-cresol L⁻¹ completely inhibited the germination (Fig. 1C and D). Spinach and chickpea seeds were more resistant and exhibited partial germination even at 400 mg *o*-cresol L⁻¹, whereas at 800 and 1200 mg L⁻¹ there was marked reduction in GP and SV (not shown in the figures). Green gram, pumpkin, red chili, and tomato seeds were more resistant to *o*-cresol even up to a concentration of 1200 mg L⁻¹ (not shown in figures).

Higher phytotoxicity of *o*-cresol on local Chinese vegetables has been reported by Guan and Poon [4]. Among seeds of 7 Chinese vegetables exposed to *o*-cresol all of them except *Cucumis melo* var. conomon Makino were found to be highly sensitive. The sensitivity to *o*-cresol increased in the order of *Cucumis melo* var. conomon Makino > *Raphanus sativus* > *Amaranthus mangostanus* > *Brassica juncea* coss. var. foliosa Bailey "Large mustard" > *Brassica campestris* ssp. Chinensis var. utilis. "yellow-leaf medium dowering Chinese cabbage" > *Brassica juncea* coss. Var. foliosa Bailey "Shuidong mustard" > *Brassica campestris* ssp. Chinensis var. utilis, latter 3 being highly sensitive [4]. *o*-Cresol inhibited the growth of root in all seven species. In another report okra and mustard seeds were shown to be sensitive to *o*-cresol, mustard being the most sensitive [5]. Marked reduction in germination percentage and seedling vigour was reported in these cases. There was no germination at all at and above 500 mg *o*-cresol L⁻¹. Recently, it was shown that okra seeds were the most sensitive to *m*-cresol among the 10 seeds tested, complete inhibition occurring at 200 mg *m*-cresol L⁻¹ [6]. Eggplant seeds were also susceptible, showing only very low GP and SV at 200 mg *m*-cresol L⁻¹. Unlike in the present case long-podded cowpea seeds were more resistant to *m*-cresol [6]. From the available information it could be inferred that the inhibitory effect of *o*-cresol on seed germination is more severe than that of *m*-cresol.

As the filter paper test indicated that eggplant seeds were highly sensitive to *o*-cresol it was selected for a detailed study by soil method using a narrower range of *o*-cresol concentration. As could be seen in Fig. 2A there was marked reduction in GP of seeds in cups containing 25 and 50 mg *o*-cresol kg⁻¹ soil (cup no. 2 and 3, respectively) as compared to the control soil without *o*-cresol (cup no. 1). Apparently, germination was very low in the case of 100 mg *o*-cresol kg⁻¹ soil and there was no germination in cups 5 and 6 containing 150 and 200 mg *o*-cresol kg⁻¹ soil, respectively. Fig. 2B depicts representative samples of seedlings/seeds of eggplant exposed to different amounts of *o*-cresol where it could be noticed that it is the root that was getting more severely affected than the shoot by *o*-cresol. The seedlings also showed negatively geotropic growth. In many seeds, the primary leaves did not emerge out of the seed coat. Similar abnormalities have been reported in the case of tomato seeds exposed to 2,4,5-T [23] and 3-CBA/4-CBA [24]. Peterson et al. [37] also have described inhibition of seedling development and decrease in GP of tall fescue exposed to 30 mg L⁻¹ of 2, 4, 6-trinitrotoluene (TNT) and 15 mg L⁻¹ of 4-amino-2, 6-dinitrotoluene (4ADNT). Ajisha et al. [6] also have reported reduction in both shoot and root growth in the seedlings of okra exposed to *m*-cresol.

Given in Table 1 are the GP of eggplant seeds, the mean shoot and root lengths, and the Vi of the seedlings germinated in soil containing 0, 100, and 200 mg *o*-cresol kg⁻¹ soil. Although some germination was detected just as a little sprout in cups with 100 and 200 mg *o*-cresol kg⁻¹ the growth of shoot and particularly root were very much stunted as reflected in extremely low Vi. Information on inhibition of seed germination by cresols in soil is very scanty except the report of Guan and Poon [4] on increased phytotoxicity of *o*-cresol on seed germination of seven Chinese vegetables, particularly *Brassica* spp. and that of Krishnan et al. [5] on inhibition of mustard and okra seeds by *o*-cresol. Recently, *m*-cresol has been shown to inhibit germination of okra seeds [6]. Amrutha Vijay et al. [17] have described the inhibitory effect of phenol on the germination of seeds of chickpea, mung bean, and long-podded cowpea. Phytotoxicity of phenol and 2,4,6-trichlorophenol to local agricultural plant species in China has been reported by Poon et al. [20]. Low molecular weight phenols present in olive mill wastewater and dry olive residue were shown to cause inhibition of seed

germination and other phytotoxicities in tomato, chicory and other crops [12,13]. It has also been demonstrated that phenolics and humic acids are responsible for phytotoxic allelopathic interactions in field soils and water [7–9]. Cinnamic acid and vanillin secreted by the roots of eggplant was shown to get accumulated in the rhizosphere on repeated monocropping which exerted severe auto-allelopathic effect such as inhibition of seed germination and root growth [10]. Severe inhibition of germination of *Isotoma axillaris* was described to be due to ferulic acid, catechin, phloridzin, and *p*-coumaric acid generated by boneseed (*Chrysanthemoides monilifera* subsp. *monilifera*) litter [38]. Long-term cultivation of lemon balm was shown to accumulate phenolics in the soil and exert phytotoxic effects on the growth and the essential oil yield [39].

Some works have indicated that the inhibitory effect of chemicals on seed germination is family-specific. For example, Ajithkumar et al. [24] have observed that the seeds of *Solanaceae* members such as tomato, eggplant, and tobacco were highly susceptible to 3-CBA and 4-CBA among the various crop seeds tested. Similarly, Gangadhara and Kunhi [23] have shown that the seeds of eggplant and tomato (both belonging to *Solanaceae* family) were more susceptible to 2, 4, 5-T than other seeds tested. Guan and Poon [4] also have shown increased phytotoxicity of *o*-cresol on "Shuidong mustard" and "Large mustard" belonging to *Brassicaceae* family among seven Chinese vegetables tested. Amrutha Vijay et al. [17] have demonstrated that phenol affects the germination of chickpea, mung bean and long-podded cowpea seeds all of them belonging to *Fabaceae* family. Contrarily, Krishnan et al. [5] have reported that the inhibitory effect of *o*-cresol on seed germination was not family-specific as the most sensitive seeds brassica and okra belonged to different families *Brassicaceae* and *Malvaceae*, respectively. Similarly, the seeds of okra and eggplant that were the most sensitive to *m*-cresol belonged to *Malvaceae* and *Solanaceae*, respectively [6]. In the present case also the most sensitive seeds viz. eggplant, long-podded cowpea, cucumber, and okra, all belonged to different families.

3.2 Enzyme Activities of Germinating Eggplant Seeds Exposed to *o*-Cresol

Imbibition of water by seeds during germination normally triggers various metabolic processes

including synthesis of hydrolytic enzymes, which help in hydrolysis of reserve food in to simple available form for embryo to metabolize [40]. Amylase and protease enzymes are activated normally to mobilize stored starches and proteins. An increase in these and other enzymatic activities has been reported by several workers in seeds of legumes viz. mung bean, lentil, cowpea, chickpea, pea, horse gram, moth bean, and field bean during germination reaching a maximum in 3–4 days [40–42]. Hence, it was envisaged that determination of these enzyme activities would provide some insight into the mechanism of inhibition of eggplant seed germination by *o*-cresol.

A relatively higher level of protease activity was observed after 24 h of sowing the seeds which continued to decline till day 4 and 5 then increased gradually till day 7 in the cases of control seeds and the seeds exposed to 50 mg *o*-cresol kg⁻¹ soil, respectively, the latter being slightly higher (Fig. 3A). However, in cups with 150 mg *o*-cresol kg⁻¹ soil the activity decreased continuously till day 7, although the initial activity after 24 h in this case was slightly higher than that in control seeds and those exposed to 50 mg *o*-cresol kg⁻¹ soil (Fig. 3A).

On the other hand, an enhanced activity of amylase was observed in seeds exposed to both 50 and 150 mg *o*-cresol kg⁻¹ soil than that in the control seeds (Fig. 3B), although the activity pattern was same in all the cases i.e., a steady decrease till day 5 from the higher values after 24 h of sowing, a steep increase on day 6, and then a slight decline till day 7. A similar phenomenon was observed by Sangeetha [43] where the amylase activity in germinating *Zea mays* seeds increased threefold on 5th day in seeds exposed to 200 mM NaCl + 20 mM thiourea than that in seeds exposed to 200 mM NaCl alone. However, earlier works have reported reduction in both protease and amylase activities e.g., in mustard seeds exposed to *o*-cresol [5], in okra seeds in the presence of *m*-cresol, in chickpea seeds exposed to phenol [17], in radish and green gram seeds in soil spiked with tech-HCH [21], and tomato seeds exposed to 2,4,5-T [23] and 3-CBA/4-CBA [24]. It was interesting to observe in the present case that *o*-cresol has an enhancing effect, though not very marked, on amylase and protease enzymes of the germinating eggplant seeds, which is contrary to most of the earlier reports, except in the case of maize seeds mentioned above [43]. As the response was different from earlier works,

the experiments were repeated 3 times for confirmation.

3.3 Effect of *o*-Cresol on Viability of Eggplant Seeds

TTC test revealed a gradual decrease in viability of eggplant seeds exposed to different concentrations of *o*-cresol (0 through 200 mg L⁻¹) which was proportional to the concentration of the chemical (Fig. 4). At a concentration of 175 mg *o*-cresol L⁻¹ the viability was almost zero. It could be inferred that *o*-cresol inhibits the dehydrogenases that catalyze mitochondrial respiration, thus rendering the seeds non-viable. Other works also have made similar observations e.g., mustard seeds exposed to *o*-cresol [5], okra seeds to *m*-cresol [6], chickpea to phenol [17], tomato seeds to 2,4,5-T [23] and 3-CBA/4-CBA [24], and tall fescue seeds to TNT and 4ADNT [37] all lost viability proportionate to the concentrations of the respective chemical.

3.4 Bioaugmentation of *o*-Cresol-spiked Soil and Elimination of Inhibitory Effect on Seed Germination

The potential application of bioremediation techniques such as bioaugmentation and gene augmentation with microorganisms capable of degrading the respective chemicals to soils contaminated with toxic chemicals has been clearly indicated [25,26]. Effective elimination of cresols from contaminated soils and wastewaters through bioremediation with cresols-degrading microorganisms have also been reported [5,6,27–30]. In the present study, *o*-cresol-spiked soil was bioaugmented with *P. monteilii* S-CSR-0014. Eggplant seeds were sown immediately after inoculation of the soil as well as 4 and 8 days after the bacterial amendment to provide sufficient time for the bacterium to degrade *o*-cresol. Improvement in GP and Vi of eggplant seedlings was obtained irrespective of the time of sowing the seeds, proving the efficacy of *P. monteilii* S-CSR-0014 in bioremediating the *o*-cresol-spiked soil and eliminating the inhibitory effect on germination (Table 2). The uninoculated cups containing 50 mg *o*-cresol kg⁻¹ soil showed a Vi of a meager 373.4 against 1040 in the control cups, although GP was 96%. But the same soil when inoculated with the bacterial cells showed 100% germination with improvement in Vi irrespective of the time of sowing the seeds. In cups containing 150 mg *o*-cresol kg⁻¹ soil also 100% germination was obtained in the bioaugmented soil irrespective of

the time of bacterial inoculation. However, only 90% recovery of Vi was obtained when the seeds were sown immediately after inoculation, whereas in sets where the seeds were sown 4 and 8 days after bioaugmentation full recovery of both GP and Vi were obtained (Table 2).

Table 1. Effect of different concentrations of o-cresol on germination and seedling vigor of eggplant seeds as tested in soil

o-Cresol (mg kg ⁻¹ soil)	%Gr.	MSL with SD(cm)	MRL with SD (cm)	Vi
0	100 ^c	6.82 ± 0.87 ^b	3.37 ± 1.87 ^b	1019.00 ^c
100	64 ^b	1.25 ± 0.75 ^a	1.15 ± 0.69 ^a	153.60 ^b
200	32 ^a	1.18 ± 0.62 ^a	0.95 ± 0.52 ^a	68.16 ^a

%Gr. - Germination percentage; MSL with SD - Mean Shoot length with Standard Deviation; MRL with SD - Mean Root length with Standard Deviation). Vi (Vigor Index) = (MSL+ MRL) x %Gr
DMRT was used to determine means which differ significantly at P = .05. Values with similar letters in each column do not differ significantly at the 5% level

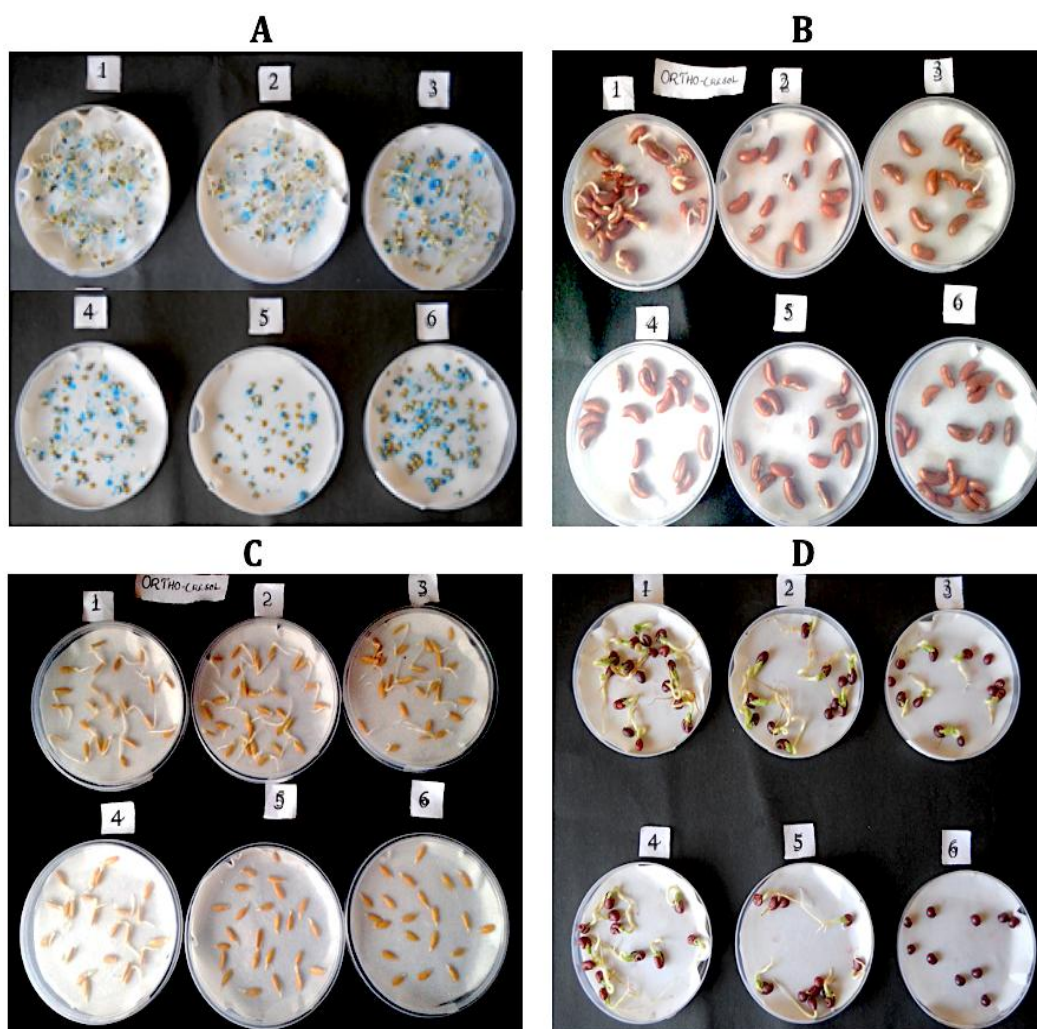


Fig. 1. Effect of different concentrations of o-cresol on germination of eggplant (A), long-podded cowpea (B), cucumber (C), and okra (D) seeds as tested by filter paper method. (Petri plate numbers 1, 2, 3, 4, 5, and 6 contained 0, 50, 100, 200, 400 and 800 mg o-cresol kg⁻¹ soil, respectively)

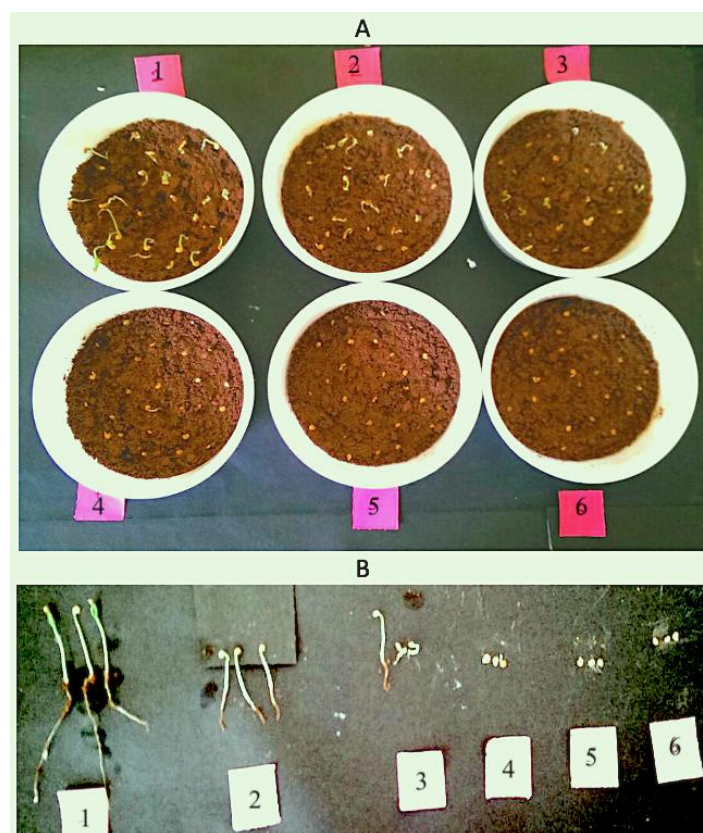


Fig. 2. (A) Effect of different concentrations of *o*-cresol on germination of seeds and seedling vigor of eggplant as tested in soil. Cup numbers 1, 2, 3, 4, 5, and 6 contained 0, 25, 50, 100, 150, and 200 mg *o*-cresol kg^{-1} soil, respectively. (B) Representative seedlings of eggplant after 7 days of growth in cups containing (from left to right) 0, 25, 50, 100, 150, and 200 mg *o*-cresol kg^{-1} soil, respectively

As already mentioned, some earlier works have indicated elimination of the inhibitory effect of chemicals by bacterial amendment of contaminated soil. Krishnan et al. [5] have shown that the inhibition of brassica seed germination by *o*-cresol could be revoked by inoculation of the soil with *P. monteilii* SHY. Ajisha et al. [6] have demonstrated that the inhibition of okra seed germination by *m*-cresol could be eliminated by bioaugmentation of the soil with *P. monteilii* S-CSR-0014, the same strain used in the present study, when the seeds were sown at least 4 days after soil amendment. Similarly, Amrutha Vijay et al. [17] have shown effective elimination of the inhibitory effect of phenol on chickpea seed germination through bioaugmentation of the soil with *P. aeruginosa* S-CSR 0013, if the seeds were sown 8 days after the bacterial inoculation. Gangadhara and Kunhi [23] also have demonstrated that bioremediation of the 2,4,5-T-spiked soil by inoculation with *B.*

cepacia AC1100 completely protected the germination of tomato seeds, if the seeds were sown 7 days after bioaugmentation. In the present case also complete recovery from the inhibitory effect of 150 mg *o*-cresol kg^{-1} soil was obtained only in seeds sown after at least 4 days after soil bioaugmentation. Probably with a little higher inoculum size a complete recovery could be obtained even if the seeds are sown immediately after bioaugmentation, which, however, need to be verified experimentally. On the other hand, Ajithkumar et al. [24] have shown effective elimination of the inhibitory effect of 3-CBA and 4-CBA on germination of tomato seeds, when sown immediately after the inoculation of the soil with *P. aeruginosa* 3mT. Similarly, Bidlan et al. [21] have reported bioremediation of tech-HCH-contaminated soil by a mixed microbial culture and elimination of the inhibitory effects on radish and green gram seed germination. Krueger et al. [22] have shown protection of pea

and soya bean seedlings from the deleterious effects of the herbicide, dicamba by inoculating the contaminated soils with dicamba-degrading bacteria. It can be presumed that in all these

cases, the inoculum size of the microorganisms would have been sufficient enough to degrade the contaminating chemicals down to non-toxic levels.

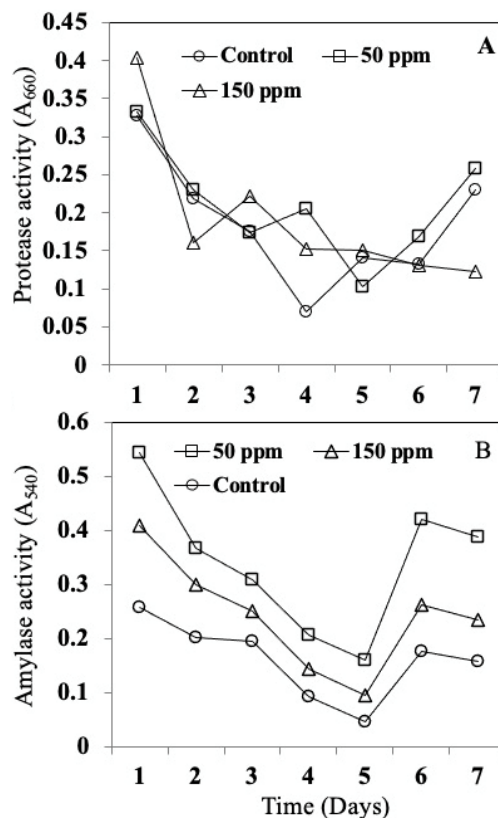


Fig. 3. Protease (A) and amylase (B) activities of germinating seeds of eggplant in the presence of 0, 50, and 150 mg o-cresol kg⁻¹ soil

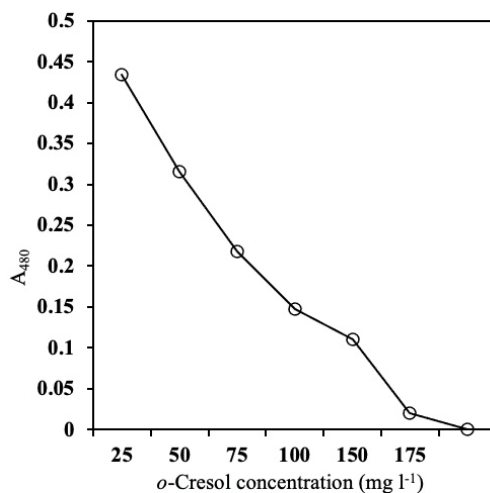


Fig. 4. Viability of seeds of eggplant exposed to different concentrations of o-cresol (0 through 200 mg l⁻¹)

Table 2. Effect of bioaugmentation of soil spiked with 0, 50, and 150 mg o-cresol kg⁻¹ soil with *P. monteilii* S-CSR-0014 on germination and seedling vigor of eggplant seeds. In all variables a set of un-inoculated cups were also maintained

Time of seed sowing	o-Cresol (mg kg ⁻¹ soil) with/without BI	%Gr	MSL with SD (cm)	MRL with SD (cm)	Vi
0 Day	Control	100 ^b	3.69 ± 1.12 ^b	6.51 ± 2.21 ^c	1020.0 ^d
	Control + BI	100 ^b	3.85 ± 0.68 ^b	6.27 ± 1.22 ^c	1012.0 ^d
	50	96 ^b	1.58 ± 0.96 ^a	2.31 ± 1.13 ^a	373.4 ^b
	50 + BI	100 ^b	4.23 ± 1.01 ^{bc}	6.17 ± 1.62 ^c	1040.0 ^d
	150	0 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.0 ^{NS}
	150 + BI	100 ^b	3.48 ± 0.89 ^b	6.02 ± 1.54 ^c	948.0 ^d
4 th Day	Control	100 ^b	3.90 ± 1.16 ^b	6.24 ± 1.20 ^c	1014.0 ^d
	Control + BI	100 ^b	4.10 ± 0.80 ^b	6.06 ± 1.29 ^c	1010.0 ^d
	50	100 ^b	2.83 ± 0.84 ^a	3.69 ± 0.69 ^b	652.0 ^c
	50 + BI	100 ^b	4.23 ± 0.85 ^{bc}	6.25 ± 1.06 ^c	1048.0 ^d
	150	68 ^a	1.03 ± 0.84 ^a	1.34 ± 0.69 ^a	161.2 ^a
	150 + BI	100 ^b	3.99 ± 0.85 ^b	6.05 ± 0.85 ^c	1004.0 ^d
8 th Day	Control	100 ^b	4.18 ± 1.28 ^b	6.13 ± 1.97 ^c	1031.0 ^d
	Control + BI	100 ^b	4.22 ± 1.02 ^{bc}	6.29 ± 1.79 ^c	1051.0 ^d
	50	100 ^b	3.12 ± 0.53 ^b	3.85 ± 1.02 ^b	697.0 ^c
	50 + BI	100 ^b	4.21 ± 1.23 ^{bc}	6.31 ± 1.41 ^c	1052.0 ^d
	150	74 ^a	2.13 ± 0.82 ^a	1.99 ± 0.92 ^a	304.9 ^b
	150 + BI	100 ^b	4.19 ± 0.62 ^b	6.21 ± 1.38 ^c	1040.0 ^d

BI - Bacterial Inoculum; %G - Germination percentage; MSL with SD - Mean Shoot length with Standard Deviation; MRL with SD - Mean Root length with Standard Deviation; Vi (Vigor Index) = (MSL MRL) x %Gr

DMRT was used to determine means which differ significantly at P = .05. Values with similar letters in each column do not differ significantly at the 5% level

3.5 *o*-Cresol Degradation and Bacterial Growth in Soil

The efficiency of the inoculated bacterium, *P. monteilii* S-CSR-0014 in degrading *o*-cresol was monitored by measuring its growth and estimating the residual *o*-cresol in the soil. The inoculated strain not only survived but exhibited an increase in cell population also in the spiked soil. In soil containing both 50 and 150 mg *o*-cresol kg⁻¹ soil the bacterium, after an initial lag of 2 days, exhibited a steady increase in growth up to day 5 in the former and up to day 7 in the latter case, which then started dwindling (Fig. 5A). In control soil without *o*-cresol there was a marginal increase in cell density up to 5 days, beyond which the cell number started decreasing (Fig. 5A). This slight growth would have been, probably, due to utilization of the small amounts of nutrients present in the soil.

Concomitant with the bacterial growth a reduction in *o*-cresol concentration was observed indicating its efficient degradation and utilization by the inoculated bacterium (Fig. 5B). In the case of 50 and 150 mg *o*-cresol kg⁻¹ soil the concentration of residual *o*-cresol was brought

down to about 5 and 30% of the initial level within 3 days, which then reached zero on day 5 and 6, respectively. The complete recovery of GP and Vi in sets, particularly that of 150 mg *o*-cresol kg⁻¹ soil, where the seeds were sown 4 and 8 days after bioaugmentation of the soil might be due to the removal of *o*-cresol sufficiently to a non-toxic level (Table 2). The disappearance of *o*-cresol from the un-inoculated soil, on the other hand, was very slow where more than 45 and 50% of the added 50 and 150 mg *o*-cresol kg⁻¹ soil remained undegraded even after 8 days of incubation (Fig. 5B). The partial disappearance of *o*-cresol in these cases might have been due to evaporation and/or binding to the soil particles (Fig. 5B).

In similar studies, Krishnan et al. [5], Ajisha et al. [6], Amrutha Vijay et al. [17] Gangadhara and Kunhi [23], and Ajithkumar et al. [24] have reported efficient degradation of *o*-cresol, *m*-cresol, phenol, 2,4,5-T and 3-CBA/4-CBA from soil bioaugmented with *P. monteilii* SHY, *P. monteilii* S-CSR-0014, *P. aeruginosa* S-CSR-0013, *B. cepacia* AC1100, and *P. aeruginosa* 3mT, respectively with concomitant growth of the bacterial strains.

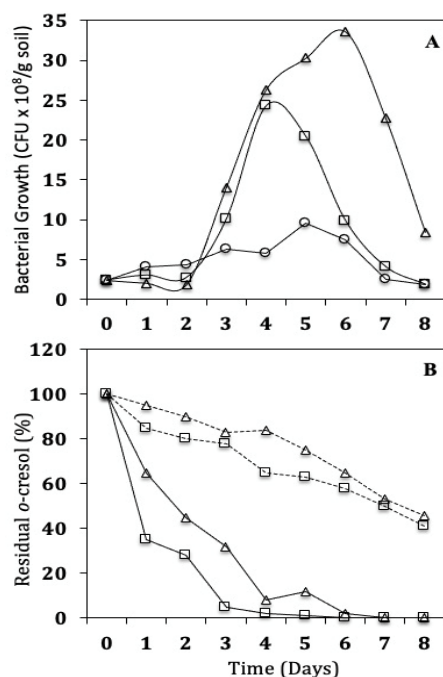


Fig. 5. (A) Survival and growth of *Pseudomonas monteilii* S-CSR-0014 in soil containing different concentrations of *o*-cresol. Keys: (-o-) Control soil; (-□-) 50 mg *o*-cresol kg⁻¹ soil; and (-Δ-) 100 mg *o*-cresol kg⁻¹ soil. (B) Degradation of different concentrations of *o*-cresol in soil un-inoculated and inoculated with the bacterium. (Keys: (-□-) 50 mg *o*-cresol kg⁻¹ soil; (-Δ-) 150 mg *o*-cresol kg⁻¹ soil; (dotted lines – un-inoculated; solid lines – inoculated)

4. CONCLUSIONS

In conclusion, the inhibitory effect of *o*-cresol on the germination of certain crop seeds, particularly eggplant and long-podded cowpea, and its deleterious effect on seedling vigor have been established. It has also been shown that these harmful effects could be eliminated effectively by bioaugmentation of *o*-cresol-spiked soil with a cresols-degrading *P. monteilii* S-CSR-0014 rendering the soil suitable for normal seed germination and healthy seedling growth. This work, however, pertains to laboratory studies only and detailed field trials in cresol-contaminated soils under natural conditions need to be carried out to ascertain the suitability of this bioremediation technique.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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