

**SEED TREATMENT WITH PHYTOHORMONES AND CROP PRODUCTIVITY. III. PHYSIOLOGICAL/BIOCHEMICAL CHANGES IN GERMINATING SEEDS AND ROOTING CHARACTERISTICS OF WHEAT (*TRITICUM AESTIVUM* L.) FOLLOWING EXPOSURE TO 2,4-D**

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**Abstract**

The 2,4-dichlorophenoxy acetic acid (2,4-D) is a commonly used herbicide. Over the past few years, its auxin-like action has been extensively exploited for modifying plant roots to achieve enhanced bacterial colonization and N<sub>2</sub> fixation. We adopted seed soaking as a mode of administering 2,4-D and observed changes in seed germination and rooting characteristics of wheat (*Triticum aestivum* L.). Soaking of seeds in an aqueous solution containing 2,4-D (0 – 200 µg mL<sup>-1</sup>) resulted in delayed or arrested seed germination. However, the number of primary roots increased dramatically and this effect was more pronounced at higher concentrations of 2,4-D. Delay in seed germination was due to decreased metabolism of seed reserves as observed by much lowered respiration rates (loss of CO<sub>2</sub>). FTIR spectroscopy revealed a relatively slow starch degradation in 2,4-D treated seeds as observed by the intensities of the characteristic absorption peaks of a broad OH band and the fingerprint region of starch. Number of primary roots increased significantly due to soaking of seed in 2,4-D solution but showed stunted growth. Scanning electron microscopy (SEM) of roots exposed to 2,4-D in the growth medium showed a strengthening of stellar system but damaged cells at the surface. Leakage of cellular material from damaged cells caused an increased colonization of roots by bacteria (revealed by SEM) and their subsequent proliferation in the rooting medium.

**Introduction**

Induction of nodulation in cereals has been a long-standing ambition of scientists with the objective to make them meet at least part of their N requirements through root-associated N<sub>2</sub> fixation. This objective has been achieved to an extent that root modifications can now be induced in cereals such as wheat, rice, and maize (Tchan & Kennedy, 1989; Kennedy & Tchan, 1992; Ridge *et al.*, 1993; Francisco & Akao, 1993; Kennedy *et al.*, 2000; Azam & Lodhi, 2001; Kennedy & Islam *et al.*, 2001). The most common approach used in these studies has been to expose the germinating seed to small concentrations of 2,4-D (a commonly used herbicide having an action similar to that of IAA). 2,4-D is a very stable compound that is not subject to breakdown by plant and is effective at a very low concentration (Ridge *et al.*, 1993). In soil, however, 2,4-D has a relatively short half-life and is rather immobile and biodegradable, average half-life being 6 days (Wilson *et al.*, 1997).

The structures formed on the roots following the exposure to 2,4-D, generally referred to as para- or pseudo-nodules are modified lateral roots (Francisco & Akao, 1993; Bender *et al.*, 1990; Kennedy & Tchan, 1992). Attempts to develop strategies to allow diazotrophic bacteria to colonize these structures and fix reasonable quantities of

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atmospheric N have produced results that are variable and less encouraging (Shantharam & Mattoo, 1997). More recently, Kennedy *et al.* (1997) noted that the progress towards improved N<sub>2</sub> fixation in cereals could more likely be made if the dogma of legume nodulation is not used in their future development.

One approach that is commonly used by other investigators for modifying roots has been to expose roots to 2,4-D. As an alternative to this approach, the research focus in our laboratory has been to induce modifications in roots, especially to increase the number of primary roots in wheat, by soaking seeds directly in dilute solutions of 2,4-D. The optimum concentration of 2,4-D for inducing higher root number varied to some extent depending on the wheat variety. However, in almost all cases, soaking of seeds yielded higher number of primary roots rather than the development of para-nodes. In a recently published study, Gulnaz *et al.* (1999) observed that as a result of soaking there was a 60-100% increase in the number of primary roots which appeared in bunches and showed stunted growth. Additionally, presoaking of seeds has been found useful in mitigating the negative effects of salinity on wheat production.

In a preliminary study (unpublished) pertaining to optimization of conditions for seed treatment (soaking) and the subsequent greenhouse experiments showed a consistent delay of 1-3 days or a failure in seed germination. This report expands on that observation to understand the mechanism(s) involved in the delay and/or cessation of seed germination and the associated changes in rooting characteristics due to 2,4-D treatment.

## Materials and Methods

**Preparation of treated seeds or seedling material:** After surface sterilization with 95% ethanol (1-min rinsing) followed by repeated washings with sterile distilled water, seeds of wheat (*Triticum aestivum* L. cv. WL-1076) were soaked for 24 h in water containing 0, 25, 50, 100, or 200 µg mL<sup>-1</sup> of 2,4-D. Stock solution was prepared by dissolving 2,4-D in ethanol (99.8%) at 10mg mL<sup>-1</sup> and making up the solution of desired 2,4-D concentration by diluting it with distilled water. In all cases, the amount of ethanol was equilibrated to account for its effect on seed germination.

**Seed germination and seedling vigour:** After 24 h of soaking in 2,4-D solutions of varying concentrations, the seeds were transferred to sterilized filter papers moistened with sterilized distilled water and placed in pairs of Petri plates. The plates were placed in the dark at 22/16 °C day/night temperature under optimum light conditions. Observations on seed germination were recorded after 24, 28, 46, and 52 h. Data on number of roots, and root and shoot length were recorded 7 days after planting soaked seeds on Petri plates lined with moist filter paper.

**Respiratory activity during seed germination:** Five seeds soaked for 24 h in water containing 0, 25, 50, and 100 µg mL<sup>-1</sup> 2,4-D were transferred into triplicate 250-mL sample chambers of a fully computerized closed circuit Micro-Oxymax Respirometer System (Columbus Instruments Inc., Columbus, OH) equipped with an expansion interface, a condenser and a water bath. The sample chambers were placed 25 °C in a water bath and connected to the Micro-Oxymax Respirometer. The experiment was carried out for 260 h and the cumulative amount of CO<sub>2</sub> in reaction chambers was recorded every 26 h.

**Table 1. Effect of soaking of seeds on seed germination and seedling vigour.**

2,4-D (ppm)	Seed germination, % of total planted at:				Number of roots	Root length (cm)	Shoot height (cm)
	24-h	28-h	46-h	52-h			
0	43	63	78	92	5	38.1	13.0
25	40	63	74	90	9	56.2	11.0
50	38	59	69	81	14	30.4	11.5
100	26	36	63	73	19	15.7	7.1
200	0	0	29	31	2	0.3	0
LSD, P=0.05	3	5	8	7	3	7.3	2.2

**Fourier transform infra-red (FTIR) analyses:** Seeds soaked in water or water containing  $100 \mu\text{g mL}^{-1}$  of 2,4-D were allowed to stay on the moist filter paper for 24-h for initial germination (seeds were considered germinated when the plumule emerged). The germinated seeds were macerated into a fine paste which was freeze-dried after physically removing the coarse material that also contained testa. The KBr pellets of the material were prepared as described by Gould *et al.*, (1990). Starch analyses were performed with a KVB/Analect RFX-75 FTIR spectrometer.

**Scanning electron microscopy:** One-week old seedlings raised on moist filter paper were transferred to unsterilized distilled water with ( $10 \mu\text{g mL}^{-1}$ ) or without 2,4-D. After one week of growth under natural light (temp.,  $22^\circ\text{C}$ ), the roots were harvested and dehydrated for 10 minutes each in a series of ethanol solutions (50, 60, 70 80, and 100%). Samples were mounted on aluminum stubs with graphite tape vacuum-coated with gold palladium and scanned (JOEL model 1200 EX scanning electron microscope) for surface morphology, general appearance and for any visible bacterial growth. To view a fractured surface, sample was quickly frozen in liquid nitrogen and cut with a sharp knife before coating.

**Microbial growth as a measure of root damage by 2,4-D:** The root damage is usually accompanied by an increased turbidity in the root medium due to release of cellular material and consequently bacterial growth utilizing secretory products as a carbon source. To measure such an effect, ten seedlings (one-week old) raised on moist filter paper in the Petri plates were transferred to distilled water containing 0, 0.5, 1.0, 2.5, 5 or  $10 \mu\text{g mL}^{-1}$  2,4-D. The base of the flasks was covered in such a way as to keep the rooting portion under dark conditions. During 15 days of seedling growth, optical density of the rooting medium was regularly measured at 560 nm as an index of the release of carbonaceous material from the roots and bacterial growth. At 15<sup>th</sup> day, aliquots of the rooting medium were counted for colony forming units of bacteria using dilution plate method and triptose soy agar medium.

## Results and Discussion

The 2,4-D treatment led to a delay in seed germination. The delay was more prominent at increased 2,4-D concentrations (Table 1). Data further indicated that both the rate and extent of seed germination were significantly retarded as a result of 2,4-D treatment. Some earlier studies also exhibited a retardation or failure of seed germination following soaking in 2,4-D solution (Gulnaz *et al.*, 1999a,b; Azam & Lodhi, 2001). In treated seeds, failure to germinate was accompanied by the release of cellular material in the growth medium, which provided suitable conditions for microbial growth. Data on respiratory activity of the seeds/seedlings revealed a significant decrease in the net accumulation of  $\text{CO}_2$  due to 2,4-D treatment (Fig. 1). At a concentration of  $25 \mu\text{g mL}^{-1}$ , 2,4-D enhanced the rate of respiration, while at concentrations  $>25 \mu\text{g mL}^{-1}$ , a consistent decrease was recorded.

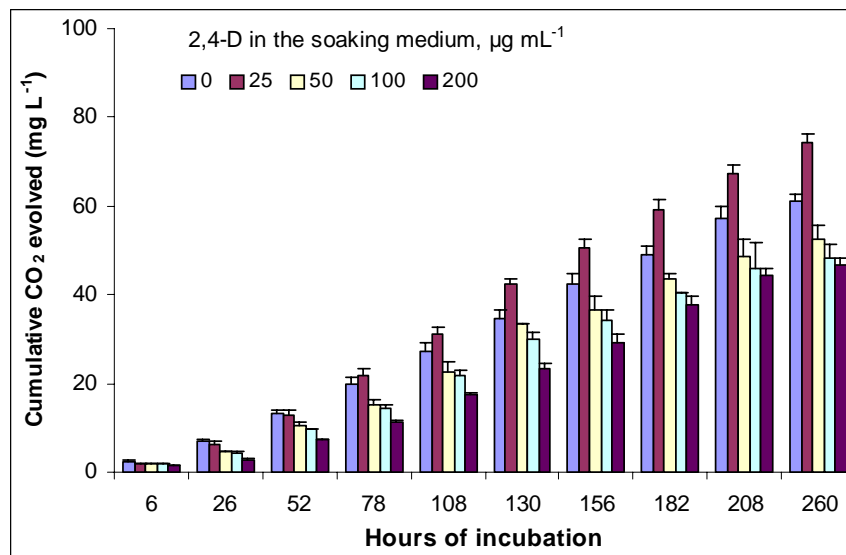


Fig. 1. Cumulative amounts of CO<sub>2</sub> evolved over 260 hours from germinating seeds following 24 h soaking in water containing 0, 25, 50, 100 or 200 µg mL<sup>-1</sup> of 2,4-D.

The FTIR spectra of the control seeds as compared to the 2,4-D treated seeds showed a small but noticeable difference in absorption intensities in two pertinent spectral regions (Fig. 2). These are the O-H (3386 cm<sup>-1</sup>, H-bonded) and C-C-O (1034 cm<sup>-1</sup>) stretching vibrational modes of the starch component. The characteristic O-H stretching frequency of the control seed (Fig. 2A) showed an attenuated intensity (10-12 %) lower than that of the 2,4-D treated seeds for same sample size (Fig. 2B). A corresponding diminution in absorption intensity (7.0-8.6 %) in the untreated seeds was also noticed in the C-C-O stretching region of the starch component. From these spectral data, it could be inferred that a relatively higher loss of starch content had occurred in the control seeds due to unimpaired metabolism compared to the treated seeds.

The number of primary roots increased from 5 in control to 19 at 100 µg mL<sup>-1</sup> of 2,4-D. At 200 µg mL<sup>-1</sup> of 2,4-D, only 2 small rudimentary roots were observed (Table 1). Francisco *et al.*, (1993) found that 2,4-D treatment produced much higher number of primary lateral roots of smaller size and the effects was proportional to the concentration of 2,4-D. In contrast, data presented here indicated a significant increase in the number of primary roots rather than primary lateral roots. It has been shown that the modification in lateral roots could be achieved at lower concentrations of 2,4-D (Bender *et al.*, 1990), but at concentrations exceeding 5 x 10<sup>6</sup> M only few or no lateral roots were produced. In the present study, roots generally appeared in bunches that were preceded by callus formation. This effect was more evident at 50 and 100 µg mL<sup>-1</sup> of 2,4-D (Fig. 3). Formation of such structures during germination of wheat seeds has been reported earlier (Gulnaz *et al.*, 1999). Ridge *et al.*, (1993) has attributed this to the fusion of multiple meristems, whereas, Nishimura & Maeda (1982) observed that 2,4-D treatment altered the position, polarity, and axis of cell enlargement of the lateral root primordia causing them to develop such structures. In spite of the increase in the number of primary roots, the cumulative root length in the present study decreased significantly due to seed treatment; the effect being more adverse at 100 and 200 µg mL<sup>-1</sup> of 2,4-D.

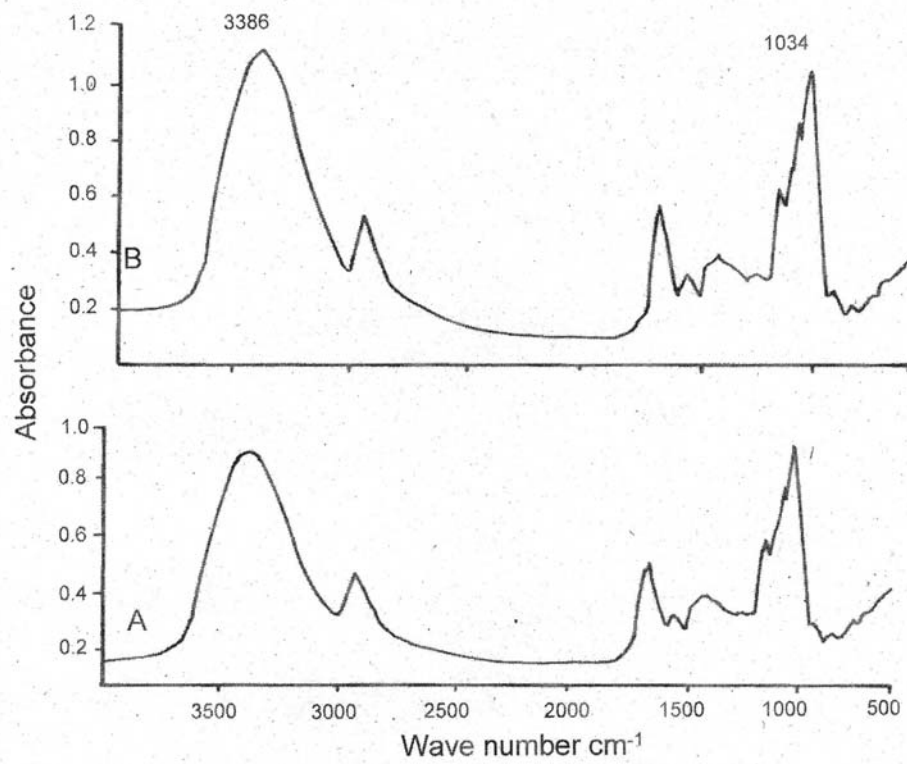


Fig. 2. FTIR spectrum of seeds soaked for 24 h in water (A) or water containing 100  $\mu\text{g mL}^{-1}$  of 2,4-D (B).

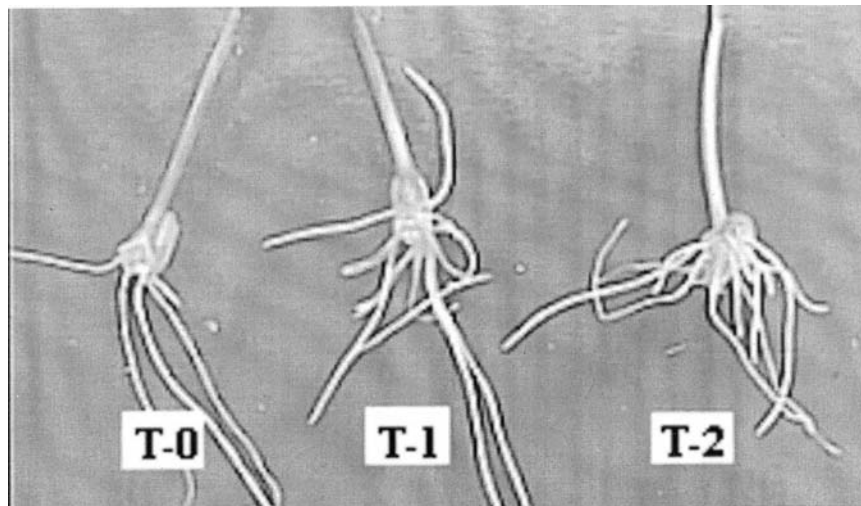


Fig. 3. Effect of seed soaking in 2,4-D on number and proliferation of primary roots. T-0, control (no 2,4-D); T-2 and T-3, 50 and 100  $\mu\text{g mL}^{-1}$  of 2,4-D, respectively.

Roots of seedlings that were raised on moist filter paper and subsequently placed in to water that contained different concentrations of 2,4-D showed significant changes in surface morphology, anatomy, cellular integrity, and bacterial colonization. Root hair development was significantly inhibited by 2,4-D (Fig. 4A,B), while bacterial colonization increased to a significant extent (Fig. 4C,D). However, damage to the surface cells was not commensurate with the root anatomy. Exposure of roots to un-aerated rooting medium without 2,4-D resulted in the loss of cell structure (conducting tissues), while presence of 2,4-D had a stabilizing effect on these tissues (Fig. 4E,F). The release of cellular material and a concomitant bacterial growth in treated seeds was supported by an increase in optical density of the rooting medium with time (Fig 5). Over a 15 days growth period, optical density of the rooting medium increased consistently with time and with the concentration of 2,4-D. The root damage appeared to start after 10 days of growth and increased sharply during the subsequent 5 days. A simultaneous increase due to 2,4-D was also observed in the number of colony forming bacterial cells i.e., from  $4 \times 10^7$  in control and  $640 \times 10^7$  at  $200 \mu\text{g mL}^{-1}$  of 2,4-D. These observations would suggest a damaging effect of 2,4-D on plant roots leading to enhanced bacterial proliferation that may serve as a prelude to microbial infestation of roots and their entry into the root interior. Treatment of seeds with 2,4-D is reported to show a positive correlation with increased internal bacterial colonization in wheat roots (Kennedy & Tchan, 1992). Christiansen-Weniger (1992) found considerable stimulation of  $\text{C}_2\text{H}_2$  reduction in 2,4-D treated wheat seedlings. According to Kennedy & Tchan (1992), adequate colonization is related to adequate carbon substrates diffusing from the root surface. Hence, one of the mechanisms whereby 2,4-D enhances bacterial colonization of roots may be through an increase in rhizodeposition by the plants probably due to cells becoming leaky as observed in the present studies. Using  $^{14}\text{C}$ , Elencheyhian & Panwar (1997) found higher translocation of photosynthates to the rhizosphere due to the 2,4-D treatments.

Kennedy & Islam (2001) proposed that 2,4-D has an effect akin to bacteria that produce cellulases and polygalacturonases. According to these authors, these enzymes are involved in loosening as well as breaking intercellular bonds. The cells thus released will add to the amount of rhizodeposition with a consequent increase in bacterial colonization of roots. Hurek *et al.*, (1994) found that apical region of the root behind the meristem was the most intensively colonized by bacteria. Indeed, the release of root border cells or root cap cells (BRD) is a common feature in most crops including wheat and serve as substrate/bait for bacteria/pathogens (Hawes *et al.*, 1991). Presence of 2,4-D in the rooting medium may facilitate the release of such cells. Francisco *et al.*, (1993) observed 2,4-D to cause sloughing off of the epidermis as the cells proliferated from the actively dividing apical and adjacent lateral meristems. Schloter & Hartmann (1998) found effective colonization of root tip cells by different bacteria probably because of root cap cells or root border cells serving as the carbon source. Christiansen-Weniger (1992) showed that auxin-affected portions of rice nursery are attractive infection sites for the bacteria.

An important factor encouraging bacterial proliferation and colonization will be when plant roots are exposed to stress, physical, physiological or whatever. Smart *et al.* (1995) presented evidence to suggest that insufficient bacterial biomass exists on the root surfaces of non-stressed plants grown under well-aerated conditions to quantitatively interfere with root nitrogen absorption measurements i.e., their contribution to N nutrition of plants may not be substantial. Thus any stress will make the plant exude more C into the rhizosphere with a consequent proliferation of rhizospheric bacteria. The presence of 2,4-D in the rooting medium may also be considered as a stress. Some of our unpublished

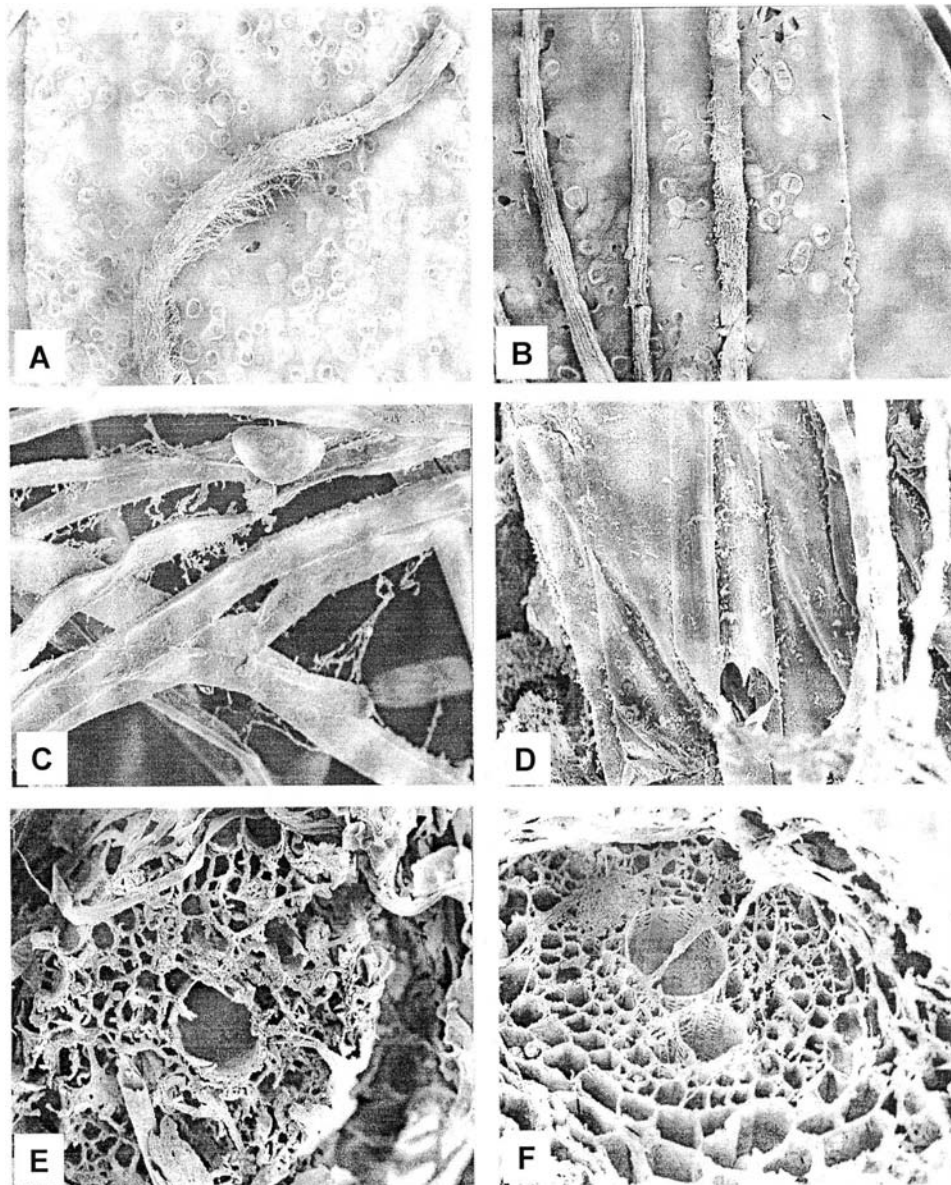


Fig. 4. Electron micrographs comparing 0 (A, C and D) or  $10 \mu\text{g mL}^{-1}$  of 2,4-D (B, D and F) in the rooting medium on i) root appearance (A and B; magnification 20), ii) bacterial infestation (C and D; magnification 1000), and iii) cut-end root anatomy (E and F; magnification 500).

work shows an accumulation of 31 kd and 34 kd proteins following seed treatment with 2,4-D. Synthesis of these proteins is reported to be caused by stresses like drought, salinity and ABA application (Eymery & Rey, 1999; Pruvot *et al.*, 1996).

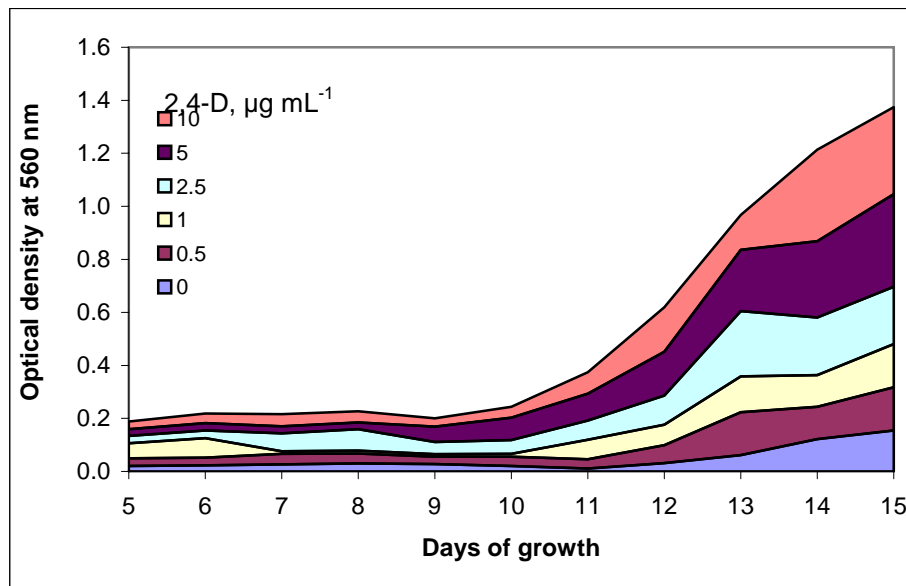


Fig. 5. Changes during 15 days in the optical density (560 nm) of the rooting medium that contained 0, 0.5, 1.0, 2.5, 5 or 10  $\mu\text{g mL}^{-1}$  2,4-D and to which ten one-week old wheat seedlings were transferred.

In addition, under low rhizospheric oxygen partial pressure (e.g., under unaerated hydroponic conditions as in the present study) roots may accumulate more soluble C (Tavaria & Zuberer, 1998 and the references therein) that may leak into the rhizosphere. Presence of 2,4-D in the rhizosphere may further enhance the release of cellular material and thus the proliferation of bacteria. In the present study, bacterial proliferation was significantly enhanced in the rooting medium containing 2,4-D and the effect was more at higher concentrations (Fig. 4). Katupitiya *et al.*, (1995) demonstrated that *A. brasilense* was more able to establish colonization on wheat roots, especially when roots were treated with 2,4-D. The 2,4-D treated plants were shown to carry more associated azospirilla than untreated plants and a dense layer of bacteria was observed covering the surface of young parts of 2,4-treated roots, nitrogenase activity was also high (Katupitiya *et al.*, 1995). Root damage by 2,4-D and its implications to nodulation in legumes and root modifications in cereals have recently been emphasized (Azam, 2001).

The results suggested that 2,4-D has a damaging effect not only on seed metabolism during germination, but also on root cells during plant growth. The damage caused to the roots and the release of cellular material may be one of the reasons for subsequent colonization and entry into the root cells of bacteria. This damage may become more severe under anoxic conditions thereby favouring the proliferation and activity of  $\text{N}_2$  fixing microorganisms. The ability of 2,4-D to cause cellular damage could be exploited further not only for making the non-nodulating legumes nodulate but also for induction of nodulation and enhancing endophytic root colonization of cereal crops. Use of 2,4-D as herbicide and its presence in the soil solution may have an effect similar to that noted in the present study following seed soaking.



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