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Degenotoxicity of Pendimethalin Contaminated Clay Soil by *Pseudomonas resinovorans* Using Anatomical, Cytogenetic and Biochemical Analysis in *Vicia faba* Plants

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Abstract

Biodegradation of the selective herbicide pendimethalin which is used to control most annual grasses was investigated in water and soil. A pendimethalin degrading bacterial isolate (designated strain E20) was isolated using enrichment technique from wheat soil previously treated with the herbicide pendimethalin on the basis of morphological, physiological and genetical (16s rDNA) tests, this bacterial isolate proved to be identified as *Pseudomonas resinovorans* E20. Results showed that the optimum pH and temperature for the growth of pendimethalin degrading strain were 7 and 30°C, respectively. Additional carbon sources (glucose, lactose, starch, carboxymethyl cellulose and phenol) decreased pendimethalin degradation. *Pseudomonas resinovorans* E20 was used to remove pendimethalin from aquatic system with half-life of 5.9 days. Pendimethalin half-life was 49.2 days in untreated mineral liquid medium as control. *Pseudomonas resinovorans* E20 was also evaluated for degradation of pendimethalin in clay soil with half-life of 5.004 days, its half-life was 66.9 days in untreated clay soil. The obtained results illustrated that pendimethalin treatment reduced all measured plant features (plant height, root length, xylem vessels diameter, thickness of phloem tissue, vascular bundle as well as vascular bundle width) in comparison to the other treatments. On the other side, treatment with *Pseudomonas resinovorans* E20 led to improving and increasing the above investigated parameters. Evaluation of the genotoxic effects of pendimethalin on the genetic material of *Vicia faba* plants revealed that, the highest abnormal mitosis percentage (63.28%) was recorded for pendimethalin treated plants. On the other hand, *Vicia faba* plants treated with the bacterial strain *Pseudomonas resinovorans* E20 decrease the abnormal mitosis percentage which reached to 21.35% in comparison with the control treatment which gave a percentage of 3.85%. This indicates the degenotoxic effect of this bacterial strain *pseudomonas resinovorans* E20. At the biochemical genetic level, the two treatments (pendimethalin and *pseudomonas resinovorans* E20) induced some changes in protein banding patterns of *Vicia faba* plants. These changes included the disappearance of three protein bands with MW's of 265, 175 and 40 KDa after treatment with the herbicide pendimethalin alone and appearance of the same bands after treatment with *Pseudomonas resinovorans* E20 bacterial strain.

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Introduction

Herbicides are potential hazards that may have toxicological and genotoxicological effects on the environment and human health (Ma, 1982). The induction of genetic damage may cause an increased incidence of genetic diseases in future generations and contribute to somatic cell diseases including cancer in the present generation (Connell, 1997). Therefore, it is very important to detect compounds or organisms that protect genetic material and avoid human exposure to pesticides. Pendimethalin is a mitotic inhibitor herbicide that can inhibit cell division in root meristems. It also inhibits microtubule synthesis, which is important for the formation of cell walls and spindle fiber. Thus, incomplete cell division and induction of multinucleated cells can occur by exposing plant cells to this herbicide. Root growth inhibition and adverse effects upon chromosomes provide indications of toxicity and genotoxicity (Rank and Nielsen, 1993).

Pendimethalin is used extensively for weed control in cotton, rice, soybean and tobacco (Smith et al., 1995). It acts by inhibiting the steps in plant cell division responsible for chromosome separation and cell wall formation. It is used before crop emergence or planting (Appleby and Valverde, 1988). Studies on terrestrial ecosystems showed that 10–20% of the herbicide vaporizes within the first week or two weeks after application (Strandberg and Scott-Fordsmand, 2004). Care should be taken to minimize excessive pendimethalin applications to the soil in order to minimize possible injury to sensitive rotation crops. It is important to develop methodologies to prevent pesticide contamination from point sources. Microorganisms can use a variety of xenobiotic compounds including pesticides for their growth, mineralize and detoxify them (Belal et al., 2008). Bioremediation is an accepted technology for accelerating the rate of cleanup of contaminated water and soil. Soil microorganisms that are repeatedly exposed to pesticides may develop new capabilities to degrade such chemicals (Vidali, 2001). There are some reports on the degradation of pendimethalin by microorganisms comprising *Azotobacter chroococcum*, *A. vinelandii*, *Bacillus circulans*, *B. megaterium Pseudomonas putida* and *Phanerochete chrysosporium* (Megadi et al., 2010; Belal and Hassan, 2013; Belal and El-Nady, 2013; Belal et al., 2014). The success of bioremediation depends not only on the high degradation ability but also on the stability of active microorganisms under varied conditions, such as changes in pH and temperature (Pattanasupong et al., 2004; Belal and Hassan, 2013; Belal and El-Nady, 2013). Therefore, it is necessary to investigate the effects of various environmental factors on the growth ability of the tested microorganisms (Pattanasupong et al., 2004).

Studying the effects of various pollutants released into the environment as a result of work done in different economic areas has become a priority in the last decades. Mode of action of various potential mutagenic factors in organisms, long-term implications of their presence on human health, represents numerous scientific research goals. Pesticides used for modern farming represent a substantial input of toxic substances in the environment, and residues present in fruits and vegetables are important risk factors for consumers (Sutan et al., 2014).

Therefore, this study attempted to use *Pseudomonas resinovorans* and evaluate its degenotoxicity effects on pendimethalin - contaminated clay soil against *V. faba* plants. Considering the negative effects of pendimethalin mentioned above, the genotoxicity of pendimethalin in the root meristem cells of *V. faba*, which was evaluated by the percentage of aberrant cells identified as having chromosomal aberrations, was also studied.

Materials and methods

The current study was carried out in Genetics Dept., Agriculture Botany Dept. and Soil Sciences Dept., Faculty of Agriculture, Kafrelsheikh Univ., Egypt during 2014 and 2015 seasons to determine the genotoxicity of the herbicide pendimethalin and degenotoxicity using *P. resinvorans* on *Vicia faba* plants.

Chemicals

Pendimethalin (N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine) standard was obtained from Ehrenstorfer (Germany). It has the empirical formula $C_{13}H_{19}N_3O_4$, a selective pre-emergent herbicide a dinitroaniline group. All other used chemicals were of analytical grade.

Microbial degradation of the pendimethalin

Media: M9-Minimal medium as mineral salt liquid (MSL) and Luria Bertani (LB) a complete medium were used through this study as described by Sambrook et al. (1989).

Isolation by enrichment culture: Enrichment cultures of microorganisms capable of degrading pendimethalin

were established from soil as described by Belal and El-Nady (2013).

Identification: The efficient selected pendimethalin degrading bacterial isolate (isolated from wheat soil treated with pendimethalin) was identified depending on morphological and physiological characteristics as described by John (1984) and 16S rDNA as follow:

Genomic DNA extraction from bacteria: DNA extraction was carried out following the CTAB method according to Azadeh and Meon (2009).

Primer design and PCR amplification: Oligo-nucleotide primers for 16S rDNA gene; 16S-1f (5'-GCTAGTTGGTGGGGTAA-3', 17 mer) and 16S-2r (5'-GCCATCTCAGTTCGGATTG-3'; 19 mer) were designed on the basis of the sequence of *E. coli* 16S gene (corresponding to positions 247 to 263 and 1291 to 1309; *E. coli* numbering system). Amplification reaction for *P. putida* (E1) was performed according to Wilems and Collins (1993).

Nucleotide sequence analysis: The PCR products were purified using a commercial kit (QIA Quick PCR purification kit (Qiagen, Valencia, CA, USA), according to manufacturer's instruction. After purification, the PCR products were sent for sequencing services at Sigma Co. Germany. The 16S gene sequences were aligned using BioEdit software versions 7.0.8 (http://www.mbio.nscu.edu/bioEdit) and searched for sequence similarity to other sequences which are available in the NCBI database at http://www.ncbi.nih.gov using Basic Local Alignment Search Tool (BLAST) algorithm. Multiple sequence alignments were performed on the selected closely related sequence accessions available using bioedit software (http://bioedit.edu/).

Phylogenetic analysis: Phylogenetic analysis was done based on the nucleotides sequences of 16S gene using mega4 or tree view software provided by the Biology Workbench Program (http://workbench.sdsc.edu/). Number of Data base JYZVMFRR015, K12FNS3P01S.

Effect of environmental factors on pendimethalin degradation by *P. resinovorans*

Experiments on the efficacy of pH and temperature on pendimethalin biodegradation were conducted in 250 ml flask containing 50 ml MSL containing 100 µg/ml of pendimethalin as a sole source of carbon. MSL medium was inoculated by 1 ml from bacterial cell suspension at 10^8 cfu/ ml. To determine the optimum pH, experiments were carried out at pH 5, 6, 7 and 8. Cultures were incubated on a rotary shaker at 30°C and 150 rpm for 10 days. To determine the effect of temperature, MSL medium with pH value of 7 was incubated at 20, 30 and 40°C under 150 rpm for 10 days. The viable cell count (cfu/ml) of *P. resinovorans* was estimated onto MSA medium containing pendimethalin. All the experiments in this study were done in triplicates.

Effect of additional carbon sources on pendimethalin degradation by *P. resinovorans*

To determine the effect of additional carbon sources on growth of the tested strain, a 50 ml MSL supplemented with 100 µg/ml of pendimethalin as a sole source of carbon. Mineral salt medium was inoculated by 1 ml from *P. resinovorans* suspension at 10^8 cfu/ml. To investigate the effect of additional carbon sources such as phenol (100 µg/ml), glucose, lactose, starch and carboxymethyl cellulose (10 gm/l), they were added to pendimethalin containing medium. Cultures were incubated on a rotary shaker at pH7, 30°C and 150 rpm for 10 days. The viable cell count (cfu/ml) of *P. resinovorans* was estimated onto MSA medium containing pendimethalin.

Biodegradation of pendimethalin by *P. resinovorans* in aquatic system

Pseudomonas resinovorans was cultured onto MSA medium supplemented with pendimethalin (100 µg/ml) for 7 days and then the growing colonies were washed with 3 ml sterilized MSL medium. The bacterial cell suspension (10^8 cfu/ml) was then used to inoculate 100 ml MSL medium containing (100 µg/ml) of pendimethalin. The cultures were incubated at 30°C, pH 7 and 150 rpm for 0, 6, 12, 18, 24 and 30 days. The percentage of degradation and the half-life of pendimethalin were determined as described afterward. Control flasks of equal volume of liquid mineral medium and pendimethalin without any microbial population were incubated in parallel at all intervals to assess abiotic loss. During the experiment, samples were collected periodically at the mentioned intervals of time for estimation of viable cell count (cfu/ml) onto MSA medium containing pendimethalin.

Bioremediation of pendimethalin contaminated soil

Clay soil without previous history of pendimethalin concentration was collected from top 12–15 cm

randomly following standard procedure and sieved through 2 mm size sieve (Gupta, 2000 and Belal and El-Nady, 2013). The physicochemical characteristics of clay soil are shown in Table 1. The experiments were conducted in 1000 g capacity pots (polyethylene pots, 20 cm inner diameter and 30 cm in depth), each having 1000 g dried clay soil. Soil was contaminated with pendimethalin (100 µg/gm soil). *P. resinovorans* was cultured onto MSA containing pendimethalin as mentioned above. One hundred ml from bacterial cell suspension (10^8 cfu/ml) was then used to inoculate 1 kg of clay soil sample, mixed well and kept under incubation for 30 days at 30°C. Throughout the incubation period, soil moisture was maintained at 40% of the field water-holding capacity. After the static incubation, 10 g aliquots from clay soil were sampled at different time intervals (i.e., 0, 6, 12, 18, 24 and 30 days) and analyzed as well as viable cell count (cfu/g soil) onto MSA medium containing pendimethalin was estimated.

The residue half-life (RL_{50}) for pendimethalin residues was calculated using the equation of Moye et al. (1987). All the experiments were done in triplicates. Control pots of equal weight of soil and pesticide without any microbial population were run in parallel at all intervals to assess abiotic losses.

Table 1. Physicochemical characteristics of clay soil.

Soil texture	Soil pH	EC (dS m^{-1})	OM (g kg^{-1})	Available element in soil (mg kg^{-1})								
				N	P	K	Cd	Ni	Pb	Mn	Cu	Fe
Clay	7.3	1.9	16	140	13.2	220	1.1	2.2	2.3	6.8	3.9	12.7

EC: soil salinity; OM: soil organic matter

Analytical procedure

Extraction and determination of pendimethalin residues were carried out as described by Jaźwa et al. (2009) at Central Agric. Pesticides Laboratory, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt. Pendimethalin residue in soil was monitored weekly after application date. At the end of that test, pendimethalin residues were determined. Soil samples were air-dried, ground and stored at room temperature prior to analysis but no more than three days. Subsamples (20 g) were extracted by shaking for one hour with 100 ml of dichloromethane-acetone mixture (9:1, v:v) on a rotary shaker. The extract obtained, was decanted by a layer of anhydrous sodium sulphate and the soil was rinsed two times with 10 ml of dichloromethane (Ambrus et al., 1981; Luke et al., 1975; Luke et al., 1981; Sadło, 1998). The extract was cleaned using florisil (Valverde-Garcia et al., 1991).

The analysis of the extract was performed using a Hewlett Packard 5890A gas chromatograph, equipped with a nitrogen – phosphorus detector (GC-NPD). The column used in this study was an HP fused – silica capillary column coated with cross-linked methyl silicone (length 25 m, ID 0.31, film thickness 0.52 µm). Nitrogen was used as both the carrier and make-up gas at a flow rate of 30 ml/min. Hydrogen was used at a flaw rate of 3.5 ml/min., and air at 120 ml/min. The oven temperature was programmed as follows: initial temperature 150°C (1 min.), rate of 10 °C/min. and final temperature 250°C. Recovery studies were carried out regularly by spiking analytical samples with stock solution of pendimethalin standard.

Statistical analysis

Data were calculated as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA). Probability of 0.05 or less was considered significant. The statistical package of Costat Program (1986) was used for all chemometric calculations.

Anatomical studies

The specimens of *V. faba* plants were taken from the basement of hypocotyl from each of the two treatments (pendimethalin and *P. resinovorans*) in addition to the control. Specimens were taken on the day 15th of sowing and fixed in formalin: alcohol: acetic acid mixture (FAA, 1: 18: 1; v/v), washed and dehydrated in alcohol series. The dehydrated specimens were infiltrated and embedded in paraffin wax (52-54°C m. p.). The embedded specimens were sectioned using a rotary microtome (Leica RM 2125) at a thickness of 8 – 10 µm. Sections were mounted on slides and deparaffinized. Staining was accomplished with safranine and light green combination, cleared in xylene and mounted in Canada balsam **(**Ruzin, 1999**)**. Ten readings from 3 slides were examined with electric microscope (Lieca DM LS) with digital camera (Lieca DC 300), and then photographed. The investigated histological features of the hypocotyl were thickness of vascular bundle and phloem tissue as well as diameter of xylem vessels.

Genotoxicity test

Cytological analysis

Cytological studies were carried out on root tips of *V. faba* plants. Ten root tips were taken from each of the two treatments in addition to the control; they were fixed in Carnoy's fixative solution (ethyl alcohol absolute and glacial acetic acid in the ratio of 3:1) for 24 h., and then stored in 70% ethyl alcohol under refrigeration. The cytological analysis was carried out using 2% aceto-carmine stain as described by Darlington and La Cour (1976). Cells were screened under a light microscope for mitotic index and chromosomal aberrations by examination of at least 3000 cells for each treatment.

Determination of mitotic index (MI) and chromosomal aberrations

Genotoxicity and degenotoxicity tests were determined as mitotic index and chromosomal aberrations for pendimethalin and *P. resinovorans* treated *V. faba* plants. The genotoxicity of pendimethalin was determined as the percentage of cells which contained chromosomal aberrations (aberrant cells). The mitotic index was calculated as the percentage of dividing cells to the total number of examined cells. Cytological abnormalities were also observed and scored. Photomicrographs of cells showing chromosomal aberrations, as well as normal mitosis, were taken using Olympus CX31 microscope. Percentages of cells showing chromosomal abnormalities, such as sticky chromosomes, laggard chromosomes, multipolar anaphases, as well as aberrant interphases (binucleated cells and micronucleus) were recorded at the appropriate mitotic stages. Both the mitotic index and chromosomal aberrations were described with descriptive statistics and then a one-way ANOVA followed by Duncan's multiple range test (1955) to compare the significance of differences between treatments and the results were considered significant at $p<0.05$.

Biochemical analysis

SDS-Polyacrylamide gel electrophoresis procedure was carried out according to Laemmli (1970). Leaves of *V. faba* plants, which were previously taken from each treatment, were used to extract water – soluble protein. Alterations in electrophoretic protein profiles in leaf samples of the treated plants were compared with those of the control to measure the mutagenic potentiality of pendimethalin and removal of the mutagenic effects after treatment with the bacterial strain *P. resinovorans*. Polypeptide bands were visualized by staining the gel with coomassie brilliant blue R-250. The obtained protein gel was scanned for band molecular weight (MW) using gelanalyzer 2010a software. Protein bands with different molecular weights were determined against PiNK prestained protein ladder with MW of 175, 130, 90, 70, 60, 50, 40, 30 20 and 15 KDa.

Results and discussion

Isolation of the pendimethalin-degrading bacteria

Initial enrichment results indicated that there was little biodegradation of pendimethalin in soil samples with no history of pendimethalin application. After successive enrichments, several mixed cultures capable of pendimethalin degradation were obtained (Fig. 1). Purified colonies (a total of 7 isolates) from these mixed cultures were tested for the ability of pendimethalin degradation as the sole source of carbon.

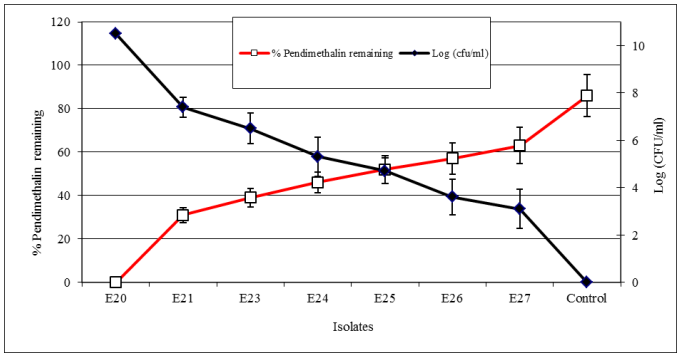

Fig. 1: The growth ability of the bacterial isolates and pendimethalin degradation in mineral salt medium.

A preliminary classification based on the morphology of the isolates revealed that the pesticide– degrading organisms belong to the group of gram positive and negative bacteria. Four of the seven bacterial isolates (E20, E21, E23 and E24) were gram-negative, motile, rods and oxidase positive. Two bacterial isolates (E25 and E26) were gram positive, motile, rods, and spore former. The best bacterial isolate (E27) was gram positive and filamentous shaped bacterium. Results in Fig. 1 showed the growth ability and degradation of pendimethalin for the seven bacterial isolates in MSL medium as a sole source of carbon. Among the seven bacterial isolates, one isolate designated as E20 achieved complete degradation of pendimethalin comparing with the other isolates. The decrease of pendimethalin was coinciding with increasing the growth rate for all the bacterial isolates. The maximum growth of E20 isolate

was achieved after 30 days of inoculation in pendimethalin supplemented MSL medium. The obtained results were compared with control treatment (uninoculated).

This bacterial isolate (E20) was identified according to morphological, physiological as well as using analysis of 16S rDNA (Fig. 2). This bacterial isolate (E20) was gram-negative, motile, rods and oxidase positive. According to the 16S rDNA analysis, the phylogenetic tree of the isolated bacterium (E20) and related bacterial species based on the 16S rDNA sequence is provided in Fig. 2. It could be clearly seen that the isolated bacterium was included in the genus *Pseudomonas* and closely related to the species *P. resinovorans*. It showed the highest sequence similarities with *P. resinovorans* ATCC14235.

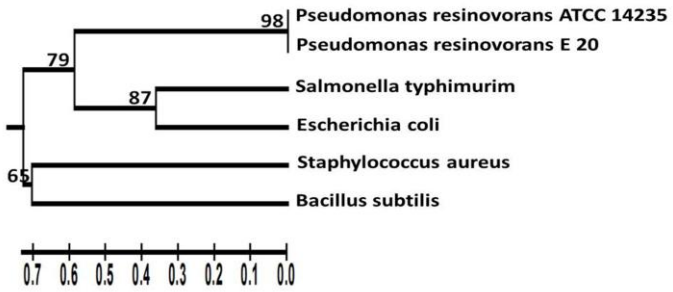

Fig. 2: Dendogram illustrating the genomic relationship among two bacterial isolates belonging to genus *Pseudomonas* revealed by UPGMA cluster analysis.

These results are in agreement with previous finding reported by (Kopytko et al., 2002; Karpouzas et al., 2005; Belal et al., 2008; Derbalah and Belal, 2008; Megadi et al., 2010; Belal and El-Nady, 2013). It was found that enrichment culture technique led to the isolation of two bacterial strains, which were able to degrade different pesticides rapidly in liquid cultures. The application of pendimethalin promotes the evolution of microorganisms that are capable of degrading this xenobiotic compound in the soil as mentioned before by Chaudhry and Ali (1988) who reported that, actinomycetes have considerable potential for the biotransformation and biodegradation of pesticides.

Effect of pH and temperature on pendimethalin degradation ability by *P. resinovorans* E20

The influence of pH on *P. resinovorans* E20 biomass growth and degradation of pendimethalin is shown in Fig. 3 (a). The highest degradation of pendimethalin was achieved at pH 7 with 45 % of degradation. After ten days of incubation, optimum pH value for *P. resinovorans* E20 biomass growth and pendimethalin degradation was found to be 7.

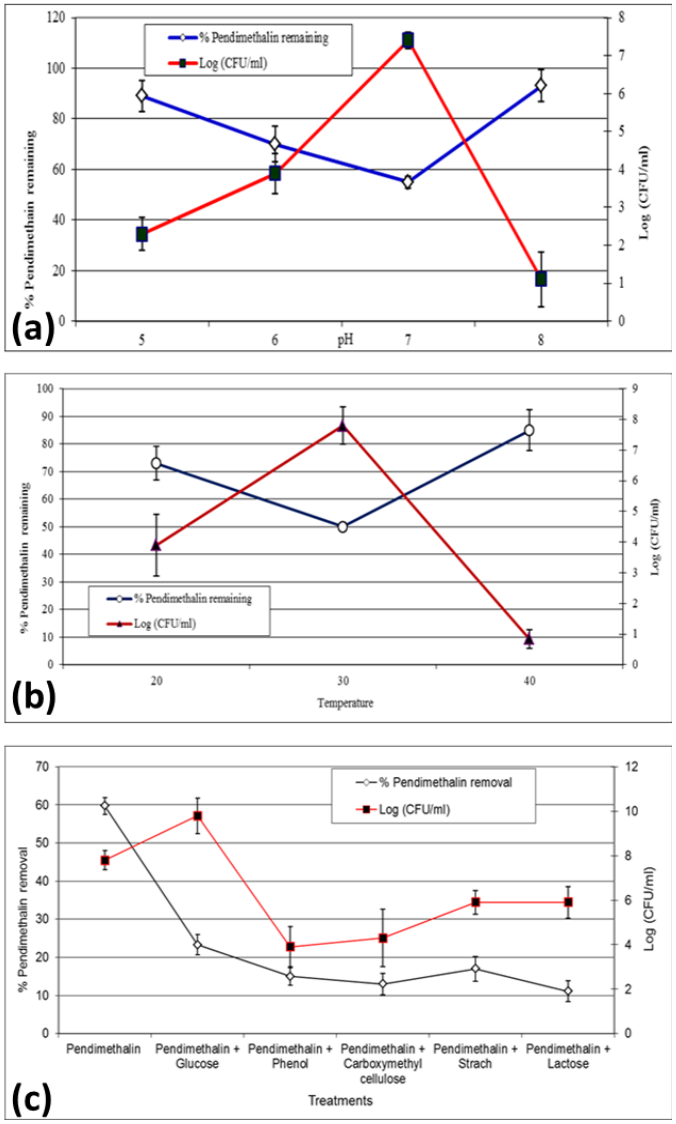

Fig. 3: Effect of pH (a), temperature (b) and additional carbon sources (c) on *P. resinovorans* (E20) biomass growth and pendimethalin degradation in mineral salt medium.

The effect of different temperatures on *P. resinovorans* E20 biomass growth and pendimethalin degradation is shown in Fig. 3 (b). Degradation of pendimethalin was occurred at 20, 30 and 40°C, while the highest degradation was achieved at 30°C, with 50% of degradation. The temperature 30°C appears to be the optimum for growth of the bacterial strain as well. The obtained results are in agreement with those reported by Belal and El-Nady (2013) who found that the optimum pH and temperature for pendimethalin degradation by *Pseudomonas putida* were 7 and 30°C, respectively.

Effect of additional carbon sources on pendimethalin degradation by *P. resinovorans* E20

Cells grown in media with additional carbon sources showed comparatively low pendimethalin degradation and biomass yield with lactose, starch, carboxy methyl cellulose and phenol. Glucose showed higher *P. resinovorans* E20 biomass than the other carbon sources and low pendimethalin degradation comparing with pendimethalin only. Phenol exhibited lower biomass and pendimethalin degradation than glucose and pendimethalin alone. The results presented in Fig. 3 (c) illustrated that the highest degradation of pendimethalin was obtained by *P. resinovorans* E20 at pH 7, 30°C for 10 days in the presence of pendimethalin only as a sole source of carbon followed by glucose, and the other carbon sources. Belal et al. (2013) found that additional carbon sources (i.e., glucose, sucrose and phenol) decreased atrazine degradation by *P. fluorescens*.

Biodegradation of pendimethalin by *P. resinovorans* E20 in aquatic system

In the present study, pendimethalin was significantly declined from the initial concentration with increasing the incubation period in the medium amended with *P. resinovorans* E20, while medium without any amendment (i.e., uninoculated control) showed less dissipation of pendimethalin (Fig. 4a). *Pseudomonas resinovorans* was able to degrade pendimethalin completely in liquid culture after 30 days of incubation revealing that pure culture application is quite promising detoxification technique through bioaugmentation. Pendimethalin half-life values were found to be 5.9 and 49.2 days in inoculated and uninoculated medium, respectively. The growth response of pendimethalin *P. resinovorans* E20 was increased gradually with the pendimethalin degradation rate increasing as shown in Fig. 4a.

This suggests that different microbial types, which may use different enzymes, have different degradation preferences. On the other hand, pendimethalin degradation percentage reached to 17% at the end of incubation time in control or non-inoculated samples. This implies the quote of pendimethalin decay due to temperature effect and volatilization (Strandberg and Scott-Fordsmand, 2004). Many authors reported that *Pseudomonas* has considerable potential for the biotransformation and biodegradation of pesticides. Members of this group are gram-negative bacteria and have been found to degrade pesticides with widely different chemical structures (Spain and Nishino, 1987; Kyria et al., 1997). The degradation of some pesticides may be attributed to the secretion of enzymes from either tested bacterial or fungal strains which are capable of degrading pesticides (Bollag and Liu, 1990).

With regard to biological metabolization, *in vitro* degradation of pendimethalin has been demonstrated by numerous authors. For instance, Kole et al. (1994) observed that 45% and 55% metabolism of pendimethalin was occurred by *Azotobacter chroococcum* after 10 and 20 days of incubation, respectively. *Azotobacter vinelandii* was isolated from a pendimethalin-treated barley rhizosphere. *A. vinelandii* utilized pendimethalin as the sole source of carbon to fix N_2 (Saha et al., 1991; Singh and Kulashrestha, 1991). Pendimethalin was degraded by oxidative N-dealkylation to yield 3,4-dimethyl-2,6-dinitroaniline and pentane. However, 6-aminopenimethalin and 3,4-dimethyl-2,6 dinitroaniline were not further metabolized because they neither supported growth of organism nor stimulated oxygen uptake. But the pentane, released by oxidative N-dealkylation of pendimethalin, was utilized as the sole source of carbon and energy for the growth of the organism. The acetylation, aryl methyl oxidation and cyclization products of pendimethalin, as reported in *Azotobacter chroococcum* (Holding and Collee, 1971) were not detected in culture filtrate of *Bacillus circulans* (Kole et al., 1994). Megadi et al. (2010) reported that pendimethalin degradation with *Bacillus circulans* was by nitroreduction and oxidative N-dealkylation via secretion of pendimethalin nitroreductase and pendimethalin N-dealkylase. The microbial degradation of dinitroaniline herbicides, pendimethalin and trifluralin has been reported to occur most often by oxidative N-dealkylation and nitroreduction. The nitro group reduction and oxidative N-dealkylation destroys the herbicidal activity of pendimethalin, leading to its detoxification (Singh and Kulashrestha, 1991; Kole et al., 1994; Megadi et al., 2010). Belal and El-Nady (2013) found that *Pseudomonas putida* degraded pendimethalin completely in mineral salt medium.

Bioremediation of pendimethalin contaminated soil by *P. resinovorans* E20

Since the previous results indicated that biodegradation successfully removed pendimethalin in aquatic system, biodegradation ability of *P. resinovorans* E20 reveals its potential for further study as a biological agent for the remediation of pendimethalin contaminated clay soil. As well as the tested microorganism in this study was a soil

borne benfecial microorganism, it may lead to reducing pendimethalin residue level in soil especially under field conditions. This investigation was further focused on the effectiveness of bioremediation with *P. resinovorans* E20, using clay soil, artificially contaminated with pendimethalin. In this case, pendimethalin was degraded faster by *P. resinovorans* E20 in inoculated clay soil (97%) compared to their respective uninoculated soil after 30 days of incubation (Fig. 4b). The abiotic loss in uninoculated clay soil was 27 and this may be due to evaporation, drift or leaching or microbial transformation. Pendimethalin half-life value was 5.004 day in treated clay soil by *P. resinovorans* E20 while it was 66.9 day in uninoculated clay soil. The application of pendimethalin increased the number of cultivable pendimethalin -degrading cells in the soil during the 30 days of incubation (Fig. 4c). On the other hand, application of pendimethalin alone decreased the population of the microorganisms compared with untreated clay soil with pendimethalin.

Biodegradation of pesticides in soil was reported with microorganisms (Belal et al., 2008; Belal and El-Nady, 2013; Belal et al., 2013). Microbial transformation/ mineralization is the most important route for pesticide degradation in soils (Luo et al., 2008; Chai et al., 2010). The size and activity of soil microbial biomass affect the rate of pesticide degradation (Beulke et al., 2005; Triky-Dotan et al., 2010). Belal and El-Nady (2013) and El-Nady and Belal (2013) found that *Pseudomonas putida* degraded completely and detoxified pendimethalin residues in soil for *Cucumis sativus* and *Echinochloa crus-galli* Plants.

A laboratory experiment simulating winter conditions showed that as much as 10% of the applied pendimethalin (0.6 mg/kg applied) was evaporated if it was applied on the soil surface. Nayak et al. (1994) investigated the effect of pendimethalin on populations of bacteria, fungi, and actinomycetes in sesame soil (sandy loam, pH 5.8, available N, P, and K 21, 23.7 and 53.75 kg/ha, respectively). It was found that pendimethalin (0.5 kg/ha) significantly reduced bacteria (61%) after 25 days but not after 50 and 75 days, at which time a slight stimulation was noted as compared with the unweeded control. Sidhu et al. (1985) and Barua et al. (1991) studied the effect of pendimethalin on populations of fungi, bacteria, and actinomycetes and reported that a significant decrease was observed on the first few days after the application, but after a period of six weeks, recovery to the level of the control was reached or almost reached.

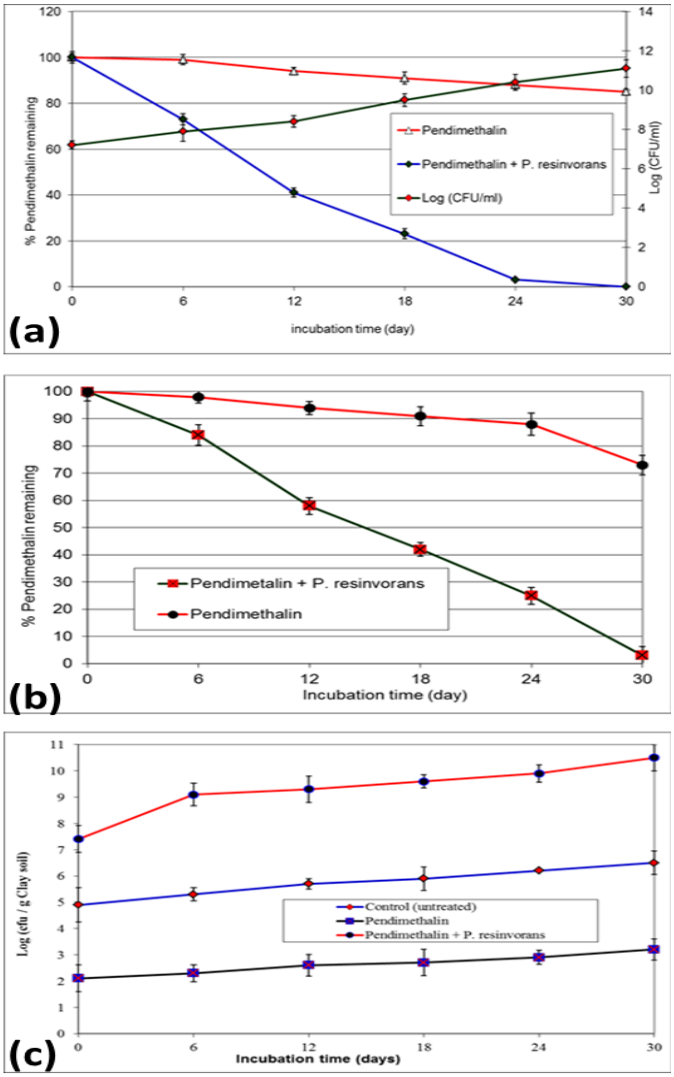

Fig. 4: (a) Pendimethalin degradation by *P. resinovorans* E20 as compared to uninoculated mineral salt medium at different time intervals. (b) Bioremediation of pendimethalin contaminated soil by *P. resinovorans* E20. (c) Bioremediation of pendimethalin-contaminated clay soil by *P. resinovorans* E20 and its effect on population of microorganisms in soil.

Phytotoxicity assessment

Vegetative characters

Data in Table 2 and Fig. 5 illustrated that, pendimethalin affects toxicity in *V. faba* plants. Where, the root elongation of *V. faba* plants treated with pendimethalin tended to decline more than that of the treated plants with *p. resinovorans* E20 and control. This was supported by the inhibiting and poisoning effects of pendimethalin. Where, application of pendimethalin treatment had a harmful impact on length of *V. faba* plant roots and plant height. In addition, it caused the highest reduction in these measured plant

parameters, the obtained results were compared with control treatment. The visible effect of herbicide is stunted root growth. So, the differences in elongation of *V. faba* roots are a very useful parameter for showing the toxicity of pendimethalin in plants. The obtained results are in agreement with previous finding reported by (Promkaew et al., 2010) on onion and maize plants. Also, Smith (2006) recorded that pendimethalin reduced the growth of both seedling weeds and crops. On the other side, *V. faba* seed treatments with *p. resinovorans* E20 have a positive impact on plant height and root length in the present investigation. Where, it led to improvement and increase in these features compared with other treatments.

Table 2. Mean values of *V. faba* plant height and root length affected by pendimethalin and *P. resinovorans* E20.

Trait	Treatments		
	Control	Pendimethalin	*P. resinovorans* E20
Root length (cm)	18	9	12
Plant height (cm)	34	14	26

Table 3. Mean values of anatomical features of *V. faba* plants affected by pendimethalin and *P. resinvorans* E20.

Treatments	Xylem vessels diameter (µm)	Phloem tissue thickness (µm)	Vascular bundle width (µm)	Vascular bundle thickness (µm)
Control	30	70	130	190
Pendimethalin	15	40	120	100
P. resinovorans E20	40	90	130	210

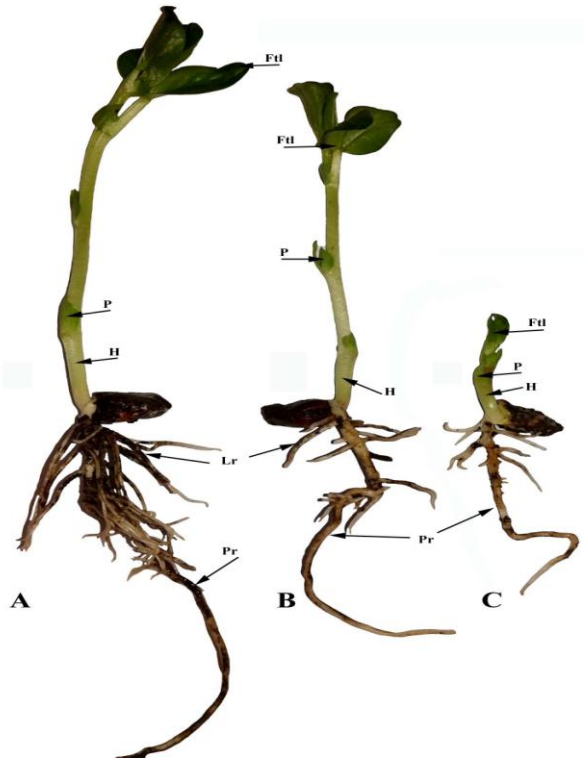

Fig. 5: Plant height and root length of *V. faba* plants under different treatments, Where: A: Control, B: *P. resinovorans* E20 and C: Pendimethalin. Ftl: First true leaf; P: Prophyll; Lr: Lateral root; Pr: Primary root; H: Hypocotyl.

Anatomical studies

The internal structure of *V. faba* plant hypocotyls is similar to stems of dicotyledonous plants. The hypocotyl structure of *V. faba* plants as seen in cross sections consists of the epidermis, ground tissue and vascular system (Fig. 6). The regions between the bundles are parenchymatous. The vascular collateral bundles arranged in complete cylinder. Data presented in Table 3 and Fig. 6 showed the effect of the remaining toxicity of pendimethalin in clay soil on some histological parameters of *V. faba* seedling hypocotyl after treatment with *P. resinovorans* E20. It has been shown from the above results that, there was a harmful impact on all investigated anatomical features of hypocotyl in pendimethalin treatment. Where, it inhibited and reduced these histological parameters. Besides that, the lowest mean values were recorded in pendimethalin treatment compared with the other treatments. Parka and Soper (1977) cleared that; dinitroaniline herbicides lead to brittleness of the stem or seedling hypocotyl and kill seedling weeds. On the other side, treatment with *p. resinovorans* E20 had a positive impact on most of anatomical characters, where it gave the highest values of xylem diameter, thickness of phloem tissue and vascular bundle; these obtained results were compared with control treatment.

Cytogenetic studies

Evaluation of the genotoxic effects of pendimethalin and degenotoxic effects of the isolated bacterial strain (*P. resinovorans* E20) on the genetic material of *V. faba* plant cells, analysis of mitotic index variations, types of chromosomal aberrations and their frequency was of great interest in this study.

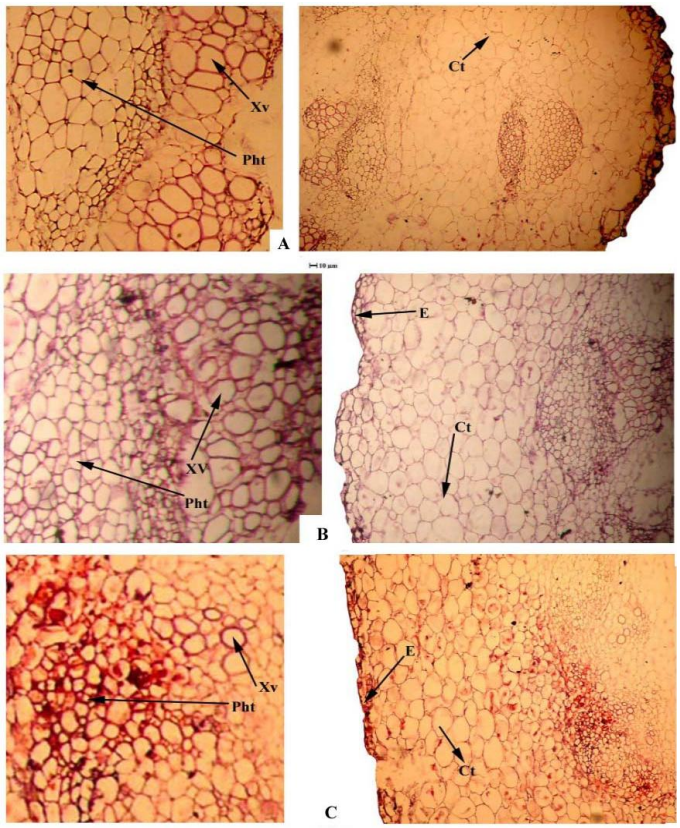

Fig. 6: Transverse sections through the hypocotyl of *V. faba* plants as affected by *P. resinovorans* E20 and pendimethalin, Where: A: Control, B: *P. resinovorans* E20 and C: Pendimethalin. E: Epidermis, Ct: Cortex tissue, Xv: Xylem vessel and Pht: Phloem tissue.

Effect of pendimethalin and *P. resinovorans* E20 on mitotic index (MI)

Decrease of MI values was associated with the type of treatment. Thus, 6.53% of the cells analyzed in control treatment were found in different stages of mitosis, while dipping the roots in soil contaminated by pendimethalin and *P. resinovorans* E20, increased the frequency of dividing cells to 9.08% and 10.30%, respectively (Table 4). The results indicated that pendimethalin was able to alter mitotic division in root tip cells. It caused a significant increase not only in the mitotic index, but also in the frequency of cells with chromosomal aberrations. Moreover, the effect of pendimethalin on mitotic cells was found to be similar to that of colchicine in the type of abnormal metaphase (C-metaphase) induction, as well as in the accumulation of metaphase cells.

Relative to the *P. resinovorans* E20 treatment, significant increase in mitotic index of *V. faba* plants was recorded. Mitotic index reached its maximum values 9.08 and 10.30 % (Table 4); which did not differ significantly, at pendimethalin and *P. resinovorans* E20, respectively. This means that pendimethalin prolonged cell division time and shortened interphase in cell division, so that division cycle shortened. Arresting cells at G1 or G2 phases of cell cycle have been mentioned by a number of studies, as a consequence of cyclin-dependent kinases (CDKs) synthesis inhibition (Polit et al., 2003; Cvikrova et al., 2003; Mitra et al., 2012). According to De Veylder et al. (2001), an important regulatory point among the different checkpoints in cell cycle was represented by the G1/S phase transition. In this transition, plant cells decided to divide (Stals and Inze, 2001), differentiate, or be inactive (Francis, 2007). The importance of this phase was considered by essential gene expression for DNA replication in the S phase of cell cycle. Therefore, G1/S checkpoint was sensitive in response to various exogenous stimuli (Nejad et al., 2012).

Effect of pendimethalin and *P. resinovorans* E20 bacteria on the chromosomal aberrations

The highest percentage of chromosomal aberrations (63.28%) was corresponding to the pendimethalin treatment (Table 4). Decrease of percentage of chromosomal aberrations in root tip cells of *V. faba* exposed to *P. resinovorans* E20 treatment (21.35%) may be the consequence of very small fraction of the cells which were dividing, and the inhibition of mitosis (Table 4). The pendimethalin and *P. resinovorans* E20 tested acted mainly in interphase, as confirmed by high frequency of Micronucleus (24.99%) and binucleated cells (11.86%) as shown in Table 5. The presence of nuclear lesions and nuclear dissolution gives cytological evidence for the inhibitory action on DNA biosynthesis during S phase of mitotic cell cycle (Mercykutty and Stephen, 1980; Akaneme and Iyioke, 2008).

Chromosome stickiness as a physiological aberration (Ping et al., 2012) was also observed in this study with the highest percentage of chromosomal aberrations (28.90) as shown in Table 5 and Fig. 7. Stephen (1979) mentioned that stickiness was a type of physical adhesion that involves mainly the proteinaceous matrix of the chromatin material. Mercykutty and Stephen (1980) reported that this stickiness might be interpreted as a result of depolymerisation of DNA during replication process, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fiber units of chromatids, and stripping of the protein covering DNA in chromosomes. As it was reported, sticky chromosomes indicated the presence of a highly toxic substance, inducing irreversible effects in the physical state of the chromatin (Östergren, 1944; Fiskesjo, 1985).

Table 4. Effect of pendimethalin and *P. resinovorans* E20 on the mitotic index (MI) and percentage of aberrant cells of treated root cells in *V. faba* plants.

Treatment	Total cells examined	Total mitosis	Mitotic index (%)	Number of dividing cells per mitotic stage (%)								Abnormal mitosis (%)
				Prophase		Metaphase		Anaphase		Telophase		
				Total	Ab.	Total	Ab.	Total	Ab.	Total	Ab.	
Control	3245	208	6.53 ± 0.29 b	25.00	-	27.36	3.39	25.40	9.43	22.24	2.13	3.85 ± 0.19 c
Pendimethalin	4307	370	9.08 ± 0.81 a	19.19	33.80	27.03	71.00	24.86	70.65	28.92	62.62	63.28 ± 2.35 a
P. resinovorans E20	5022	516	10.30 ± 0.22 a	29.46	6.58	24.81	32.81	25.19	38.46	20.54	9.43	21.35 ± 1.58 b

Values followed by the same letter did not significantly differ at 0.05 probability level.

Table 5. Percentage of different types of abnormalities detected on treated root tips of *V. faba* plants with pendimethalin and *P. resinovorans* E20.

Treatment	Percentage of different types of abnormalities										
	Sticky chromosomes	Laggards	Fragments	Bridges	C-metaphase	Micronucleus	Binucleate cells	Disturbed metaphase	Disturbed anaphase	Centric separation	Multipolar anaphase
Control	0.48	0.96	0.48	2.40	1.92	6.25	1.92	0.48	0.48	0.48	0.48
Pendimethalin	23.78	4.32	8.10	11.62	3.78	16.48	9.18	10.81	1.62	0.81	1.35
P. resinovorans E20	4.64	4.64	2.70	3.10	1.54	2.26	0.76	2.32	0.38	0.38	1.16
Total	28.90	9.92	11.28	17.12	7.24	24.99	11.86	13.61	2.48	1.67	2.99

It is likely that many of chromosomal aberrations induced by the action of various types of mutagenic agents might be due to the dysfunction of nuclear spindle. Under the action of pendimethalin, the disturbance of mitotic spindle may occur, which could cause C-metaphases and multipolar anaphase. The abnormal C-metaphases were formed as a result of the complete inactivation of division of the spindle (Fiskesjö, 1993). Consequently arrest of cells in metaphase stage might be one of the causes of mitotic inhibition. In this study, occurrence of C-metaphase, lagging chromosomes and multipolar anaphases, clearly showed the accumulated effect of pendimethalin on spindle function. Inhibition of cytokinesis following telophase was responsible for binucleated cell formation visible in the next interphase of a new cell cycle. In *Allium cepa*, some authors suggested that phragmoplast inhibition at the early stage of telophase is the responsible disturbance for binucleated cell formation (Rank et al., 2002; Majewska et al., 2003).

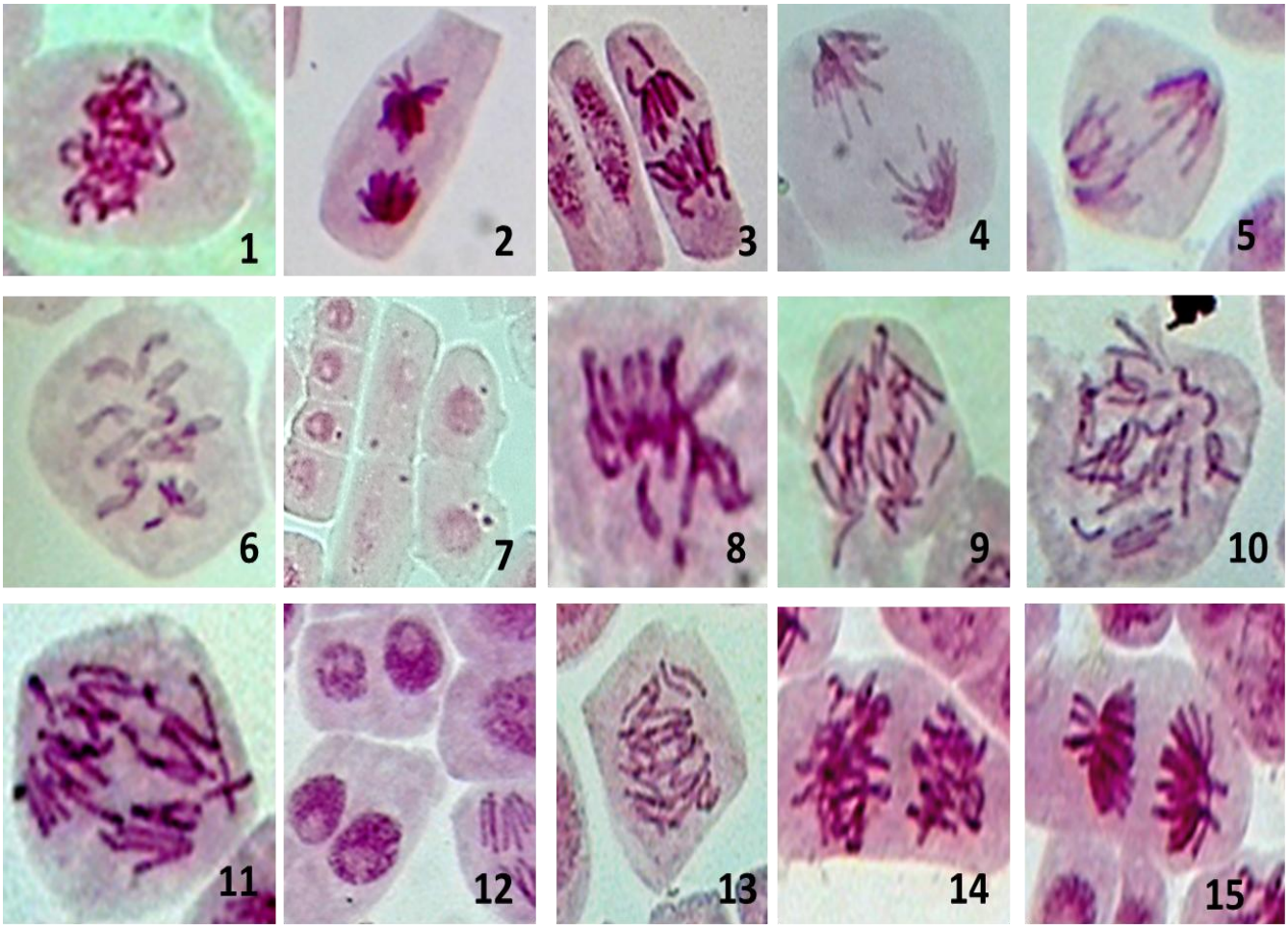

Fig. 7: Chromosomal aberrations found in mitotic cells in treated roots of *V. faba* plants with pendimethalin (1) Sickness prophase; (2) Sticky telophase; (3) Laggard chromosome; (4) Fragments; (5) Single bridge; (6) C-metaphase; (7) Cell showing one and two micronuclei; (8) Disrupted metaphase; (9) Disrupted anaphase; (10) Centric cebaration; (11) Multipolar anaphase; (12) Binucleate cell; (13) Chromatide break; (14) Duplication; and (15) Star anaphase.

Effects of pendimethalin and *P. resinovorans* E20 on leaf protein of *V. faba* plants

Treatment of *V. faba* with each of the two treatments resulted in some changes in the leaf protein banding patterns as compared with those of the control. The recorded changes were expressed as variations in the number of separated bands, disappearance or appearance of certain bands and alterations in band intensity. Disappearance of some bands after treatment with pendimethalin and appearance of the same bands after treatment with *P. resinovorans* E20 extract was of specific interest recorded in the present study.

Leaf proteins profile of the control treatment was found to be nearly identical the treatment with *P. resinovorans* E20 (the majority of bands were monomorphic) as shown in Fig. 8. The bands with MW′s of (265, 175 and 40 KDa) were polymorphic, since they did not appear in pendimethalin treatment (lane 3 in Fig. 8). Disappearance of some bands after treatment with the pendimethalin in this study, could be traced back to the induction of two mutational types i.e. gene mutation and chromosomal aberrations.

Barakat and Hassan (1997) in their study on concluded that induction of bridges, laggards and micronuclei would lead to a loss of some of the genetic materials; therefore, some of the electrophoretic bands might disappear due to the deletion of their corresponding gene(s). Also, the disappearance of these bands could be explained on the basis of mutational event at the regulatory genes that prevent or attenuate transcription (Muller and Gottschalk, 1973). On the other hand, the same number of protein bands was recorded in protein profile of leaves produced by the treatment with *P. resinovorans* E20 (lan 2 in Fig. 8). Thus, three polymorphic protein bands were appeared by application of the different treatments.

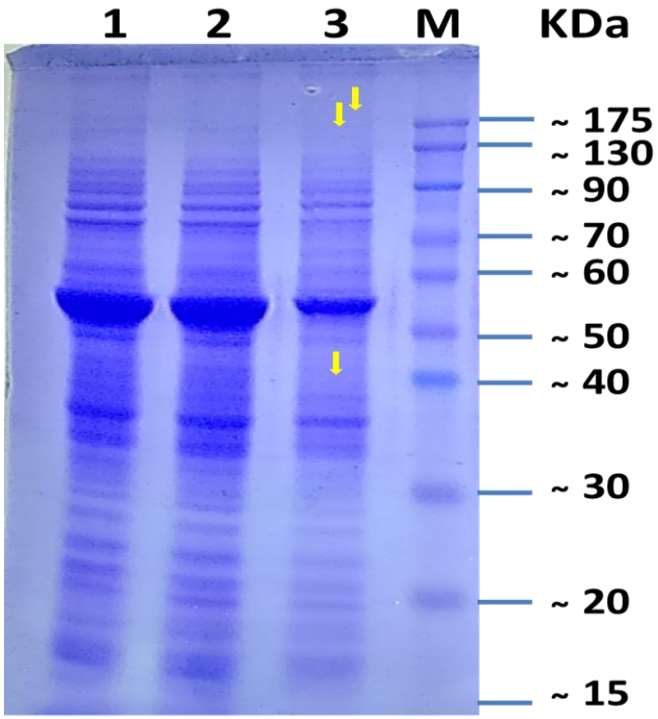

Fig. 8: SDS–PAGE banding patterns of total proteins extracted from *V. faba* leaves pretreated with pendimethalin and *P. resinovorans* E20. Lane M: molecular weight marker. Lane 1 for control and lanes 2 and 3 for *P. resinovorans* E20 and pendimethalin treated plants, respectively.

Appearance of new bands may be explained on the basis of mutational event at the regulatory system of an unexpressed gene (s) that activates them (Abdelsalam et al., 1993; El- Nahas, 2000).

Considering bands intensity, remarkable variations between the control and the two treatments were observed. Comparing with the control, there was a decrease in the intensity of all bands in protein profile produced by pendimethalin treatment, especially the band with molecular weight of 55 KDa. Whereas, no bands were increased in their intensity as a result of treatment with *P. resinovorans* E20 compared with control. This conclusion is in agreement with those of Gamal El-Din et al. (1988) who noticed that increasing the number of genes encoding for the different protein subunits through doubling of chromosome number from 12 to 24 in *V. faba* caused an increase in band intensity.

Conclusions

The present study proved that the herbicide pendimethalin caused increase of the mitotic index in *V. faba* plants, and chromosomal aberrations. The concentrations used in the field were high and could be more harmful for the end-receptors by food-chain. *Pseudomonas resinovorans* E20 was used in this study in attempt to decrease the genotoxic effect of pendimethalin. Our results proved this bacterial strain has a degenotoxic effect against this herbicide. Therefore, it is preferable to use *Pseudomonas resinovorans* E20 as possible with pendimethalin herbicide to prevent or reduce its mutagenic and genotoxic effects in target and non-target organisms.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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