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Effects of *Glomus mosseae* on the toxicity of heavy metals to *Vicia faba*

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Abstract: A glasshouse pot experiment was conducted to investigate effects of the arbuscular mycorrhizal fungus *Glomus mosseae* on the growth of *Vicia faba* and toxicity induced by heavy metals (HMs) (Cu, Zn, Pb and Cd) in a field soil contaminated by a mixture of these metals. There was also uninoculation treatment (NM) simultaneously. Mycorrhizal (GM) plants have significantly increased growth and tolerance to toxicity induced by heavy metals compared with NM plants. P uptake was significantly increased in GM plants. Mycorrhizal symbiosis reduced the transportation of HMs from root to shoot by immobilizing HMs in the mycorrhizal, shown by increasing the ratios of HMs from root to shoot. Oxidative stress, which can induce DNA damage, is an important mechanism of heavy metal toxicity. GM treatment decreased oxidative stress by intricating antioxidative systems such as peroxidases and non-enzymic systems including soluble protein. The DNA damage induced by heavy metals was detected using comet assay, which showed DNA damage in the plants was decreased by the GM treatment.

Keywords: arbuscular mycorrhizal fungi (AMF); metal contamination; metal toxicity; DNA damage

Introduction

Metal contamination of soils and subsequent uptake by plants represents one of the major environmental problems in China. Soil contamination with heavy metals (HMs) is derived from various sources, such as atmospheric deposition, the use of sewage sludge in agricultural land and so on. The accumulation of heavy metals in agricultural systems contributes not only to the detrimental effects on soil ecosystems but also to potential human health risks via the accumulation of HMs in food chains (McLaughlin and Songh, 1999).

How to improve the growth condition of plants in soils contaminated by HMs is becoming one of the focuses of environmental research. Previous studies have indicated that HM uptake by plants is related not only to the HM concentrations in soils but also to other factors, such as the symbiosis of plants with microorganisms especially with arbuscular mycorrhizal fungi (AMF), the species of AMF, plant genotypes etc. It has been shown that most plants can be colonized by AMF and that AMF can play an important role in alleviating the HM phytotoxicity (Ouziad *et al.*, 2005; Leyval, 1997; Zhu *et al.*, 2001; Chen *et al.*, 2004; Zhang *et al.*, 2005). In soil contaminated by HMs, mycorrhizal symbiosis usually reduces the uptake and transportation of HMs, leading to decreased concentrations of these metals in the aerial plant parts, and consequently result in beneficial effects on plant growth, which has been reported for Zn (Hetrick *et al.*, 1994). In the past many researches had focused on a single pollutant by adding one of the HMs to non-contaminated soils (Chen *et al.*, 2004). However, contaminated soils often contain more than two pollutants (mixed contamination), and little is known about the effects of AMF on plants, growing on soils contaminated with high concentrations of co-existing HMs. It is also not clear whether the physiological or biochemical characteristics of host plants are affected by AMF colonization exposed to a mixture of Cu, Zn, Pb and Cd in soil. HMs can induce oxidative stress, which leads the DNA damage in plants, and there were few papers about the effects of AMF on DNA damage in host plant induced by HM mixtures. Single cell gel electrophoresis (SCGE), which is also called comet assay, can be used to assess the DNA damage induced by the chemicals in the environments (Lin *et al.*, 2005a, b). This technique offers a simple, fast and reliable method to test DNA damage in plants induced by HMs with or without arbuscular mycorrhizal (AM) symbiosis.

Therefore, to investigate the possible mechanisms of AMF-mediated alleviation of toxicity induced by HMs, a pot culture experiment was conducted under greenhouse conditions with the following aims: (1) to examine the effects of AMF on the growth of *Vicia faba* in soil contaminated by HMs; (2) to measure the uptake and transportation of HMs by *Vicia faba* in contaminated soil in relation to AMF; (3) to detect physiological and biochemical characteristics, especially DNA damage in *Vicia faba* induced by HMs in the soil and the effects of AMF.

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1 Materials and methods

1.1 Growth medium

The field soil sample (paddy soil) used in all the experiments was collected from Fuyang, Zhejiang Province, China. Soil was sampled from the surface layer (0—20 cm) of cultivated fields which were contaminated by the HMs such as Cu, Zn, Pb and Cd from local smelting factories. The soil was sieved to pass a 2-mm mesh, autoclaved (121℃, 2 h) to eliminate indigenous AMF, and then air-dried. Soil pH (soil: water = 1:2.5) was 6.3 before sterilization. The soil was digested with aqua-regia (the ratio of HNO_3 : HCl is 1 : 3) at 160℃ for 48 h and the concentrations of Cu, Zn, Pb, Cd and phosphorus (P) in solutions were determined by inductively coupled plasma-atomic emission spectroscopy(ICP-AES). Bio-available fractions of Cu, Zn, Pb and Cd in soils were extracted using a solution containing 0.005 mol/L DTPA (diethylene triamine pentaacetic acid), 0.01 mol/L $CaCl_2$ and 0.1 mol/L TEA (triethanolamine) at pH 7.30. Plant available P was extracted by 0.5 mol/L $NaHCO_3$ solution and determined colorimetrically by the vanadomolybdate method. In all treatments basal nutrients in solution were mixed with the soil. In each pot nutrient was added at rates (kg^{-1}) of 684 mg N (NH_4NO_3), 300 mg K (K_2SO_4) and 180 mg Mg ($MgSO_4$), 300 mg Ca ($CaCl_2 \cdot 2H_2O$) to the soil.

1.2 Plant growth conditions

Seeds of *Vicia faba* were surface-sterilized with 10% (v/v) H_2O_2 for 10 min and immersed in deionized water for 24 h. They were then germinated on moist filter paper until the radicles appeared. Uniform seedlings were selected to use in the experiments. The *Vicia faba* was inoculated with *Glomus mosseae* (GM) or left noninoculated (NM). Plants were grown in round plastic pots containing 460 g soil plus 40 g inoculum (*Glomus mosseae*, BGC, XJ01) for the GM treatment or 460 g soil plus 40 g sterilized isolate for the NM treatment. The inocula were homogenized with the soil. Three pregerminated seeds were sown into each pot. Pots were regularly weighed to maintain soil moisture at 70% of field water holding capacity. The containers were randomly arranged in an environment-controlled growth chamber for 6 weeks with 14 h photoperiod at 280 $\mu mol/(m^2 \cdot s)$.

1.3 Plant harvest

Plants were harvested after 6 weeks of growth. After the shoots were cut off, the roots were carefully washed free of soil with tap water and then fully rinsed in deionized water. The roots were washed with 1 mmol/L $CaCl_2$ solution for 30 min to remove the heavy metals in the surface of the root.

1.4 Root colonization

The clean roots were cut into segments around 1 cm length. A randomly selected subsample of 1 g fresh root was taken for the assessment of root colonization. The roots were cleared in 10% KOH, stained with 0.05% trypan blue (Phillips and Haymann, 1970) and percent colonization was determined by the grid intersect method (Giovannetti and Mosse, 1980).

1.5 Plant biomass and HMs analysis

After being dried at 70℃ for 72 h, the dry weight of shoots and roots was determined and the samples were ground with a stainless steel mill. The plant materials were digested using concentrated nitric acid (HNO_3) with an opening-vessel method at 160℃, and the elemental concentrations were determined by ICP-AES.

1.6 Enzyme extraction and assay

Fresh root and leaf samples (about 500 mg) were ground in liquid nitrogen using a mortar and pestle. The ground samples were homogenized on ice in 10 ml solution containing 50 mmol/L potassium phosphate buffer, 1% (w/v) polyvinylpyrrolidone (pH 7.8) and were kept at 4℃ for 10 min. The homogenates were filtered and centrifuged at 4000×g for 15 min at 4℃. The Peroxidase (POD) activity was determined at 25℃ with guaiacol (Li *et al.*, 2000). In the presence of H_2O_2, POD catalyzes the trans-formation of guaiacol to tetraguaiacol (brown product). Increase in absorbance was measured at 420 nm and 30 s intervals up to 2 min, using a spectrop-hotometer. The reaction mixture contained 100 mmol/L potassium phosphate buffers (pH 6.0), 33 mmol/L guaiacol and 0.3 mmol/L H_2O_2.

1.7 Soluble protein concentration

After the POD activity was determined, the protein concentrations of the homogenates were analyzed with an ultraviolet spectrophotometer at 280, 260 nm to determine the soluble protein concentration (Li *et al.*, 2000).

1.8 Comet assay

The method of SCGE was used to detect DNA damage in the leaves of *Vicia faba* induced by combined contamination under GM and NM treatments. After harvest, the fresh leaves were washed 3 times with double-distilled water, blotted dry with a filter paper and were used in the comet assay. All operations were conducted under dim or red light to avoid DNA damage induced by the light. Plant material was placed in a 60-mm petri dish on ice and covered with 250 μl of cold 1×PBS (NaCl 130 mmol/L, Na_2HPO_4 7 mmol/L, NaH_2PO_4 3 mmol/L, EDTA 50 mmol/L, pH 7.5). Using a new razor blade, each leaf was gently sliced into pieces. The pieces were washed with the buffer by repeated pipetting using a micropipette. The nuclei were released and collected in the buffer.

The nuclei suspension was used in the alkaline comet assay, as described by Gichner and Navarrete

with some modifications (Navarrete *et al.*, 1997; Gichner *et al.*, 2003; Lin *et al.*, 2005a). After the preparation of slides they were put in freshly prepared cold alkaline buffer (300 mmol/L NaOH, 1 mmol/L Na_2EDTA, pH>13) at 4℃ to allow the DNA to denature. Electrophoresis was then conducted for 15 min at 300 mA. After electrophoresis the slides were immersed in 0.4 mol/L tris-HCl (pH 7.5) solution at room temperature for another 15 min.

Each slide was stained with 50 μl of 13 mg/L ethidium bromide and viewed with a Carl Zeiss Electromotive Microsocope (Axioskop 2 mot plus, Germany) with an excitation filter of 510—560 nm and a barrier of 590 nm. The stained DNA gives a red emission. A cool CCD captured images of the comets. For each slide 50 randomly chosen cells were analyzed.

An image analysis system, CASP was employed to measure various comet parameters such as the tail length, tail DNA, tail moment (TM) and Olive tail moment (OTM) (Konca *et al.*, 2003). The tail length, as a rough estimate of the DNA migration, was recorded in arbitrary units. Tail DNA means the percentage of DNA in the comet tail. TM is the integrated value of DNA density in the tail multiplied by the migration distance. OTM is the product of the distance (in x direction) between the center of gravity of the head and the center of gravity of the tail and the percentage of tail DNA. For each slide 50 randomly chosen cells were analyzed.

1.9 Data analysis

Results are presented as the mean ±*SE*. Data were calculated and analyzed statistically using the analytical tools of Microsoft Excel 2000. Multiple comparisons between treatments were carried out using the ascorbate acid software.

2 Results

2.1 Soil contamination status

The sampled soil contained high concentration of Cu, Zn, Pb and Cd (Table 1) indicating the soil was contaminated seriously.

Table 1 Total and extractable concentrations of Cu, Zn, Pb, Cd and P in contaminated soil

	Cu	Zn	Pb	Cd	P
Total conc., mg/kg	1481±19	2286±23	388±23	1.6±0.2	6681±76
Extractable conc., mg/kg	150±3	93±7	45±3	—	30±2

Notes: Results are expressed as the means ± *SE* (n=3); —. under the limits of the detection

2.2 Plant growth and mycorrhizal colonization

The non-inoculated *Vicia faba* was not colonized by GM, while 58% root colonization was detected in *Vicia faba* inoculated by GM. Inoculation with GM significantly increased the dry weight of *Vicia faba* (Fig.1), also produced higher root length (93.3±5.4 m) compared with non-inoculated treatments (32.0±3.9 m).

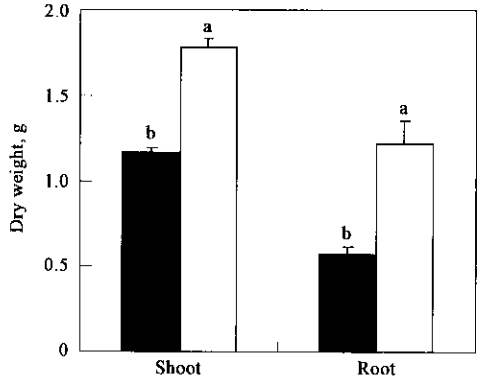


Fig.1 Shoot and root dry weight of *Vicia faba* in soil with combined contamination

Results are expressed as the mean ±*SE* (n=3); open bars: mycorrhizal (GM) plants; closed bars: uninoculation treatment (NM) plants; in each pair of open and closed bars, different letters indicate the significant difference at P<0.05

2.3 Concentrations of Cu, Zn, Pb, Cd, P in plant tissues

No significant difference was found in shoot concentrations of Cu, Zn, Cd and P in GM *Vicia faba* by GM compared with NM plant. In contrast, shoot Pb was significantly reduced by colonization of GM (Table 2). Roots of GM plants showed increase in concentrations of Cu, Zn, Pb and Cd. P concentration in roots was significantly reduced by colonization.

2.4 Translocation of Cu, Zn, Pb, Cd and P from roots to shoots

The translocation of Cu, Zn, Pb, Cd and P was determined as the ratio of HMs content in roots to that in shoots (R/S). Much higher ratios of R/S for Pb and Cd were observed in mycorrhizal plants compared with NM plants (Table 3).

2.5 Soluble protein concentration and POD activity

The soluble protein concentrations in shoots and roots of *Vicia faba* were decreased significantly by GM inoculation (Fig.2). In shoots no difference was found between the peroxidases (POD) activity of NM and GM plants but in roots the POD activity was significantly reduced in GM plants (Fig.3). In NM plants, the root POD activity was notably higher than that of shoots. In contrast, it was opposite in GM plants. Colonization by GM significantly decreased POD activity in roots. There were no significant effects of HMs on shoot POD activity because most of the HMs accumulated in the mycorrhizal roots. For the roots, much HMs entered the mycorrhizal tissues and showed no oxidative stress on the plants, which caused a decreased POD activity in the roots.

2.6 DNA damage in *Vicia faba*

Table 2 Concentrations of Cu, Zn, Pb, Cd and P (mg/kg) in shoots and roots of *Vicia faba* with mycorrhizal (GM) and without inoculation treatment (NM) in the soil

	Treatment	Cu	Zn	Pb	Cd	P
Shoot	NM	10.9±3.4^a	86±2.7^a	3.3±1.1^a	2.4±0.8^a	1991±176.6^b
	GM	15±2.0^a	116±20.2^a	0.27±0.1^b	1.9±0.7^a	2222±23.7^a
Root	NM	724±23.1^b	566±45.3^b	273.1±2^a	55.5±9.4^a	1779±44^a
	GM	997±106.4^a	831±16.2^a	370.5±19.6^a	73.5±1.8^a	1414±53.4^b

Notes: Results are expressed as the means±*SE* (n=3); different letters following the figures in the Table indicate significant difference in the means of inoculation and non-inoculated treatments by LSD at 5% level

Table 3 The ratio of Cu, Zn, Pb, Cd and P content of root to that of shoot under different treatments in the contaminated soil (*R/S*)

Treatments	Cu	Zn	Pb	Cd	P
NM	35.2±4.2^a	3.2±0.3^a	35.8±2.5b^b	11.3±1.9^b	0.4±0.07^a
GM	49.6±9.9^a	5.3±1.0^a	987.6±216.3^a	28.4±5.2^a	0.4±0.04^a

Notes: It is the same as Table 2

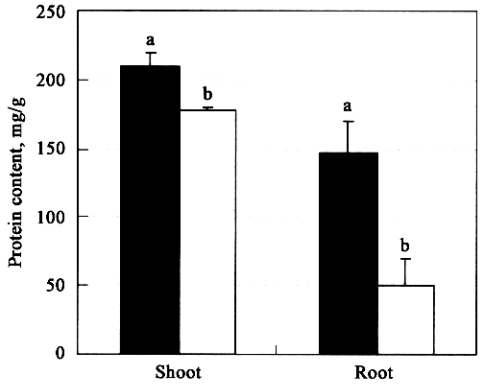


Fig.2 Shoot and root protein concentration of *Vicia faba* grown in soil with combined contamination
The same as Fig.1

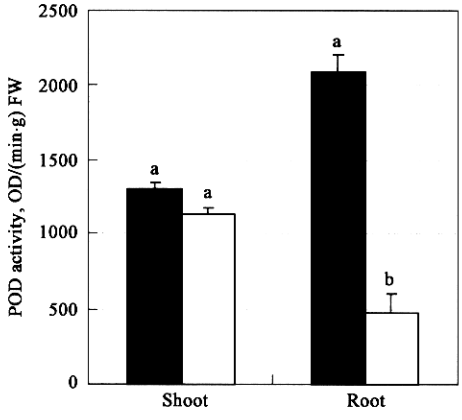
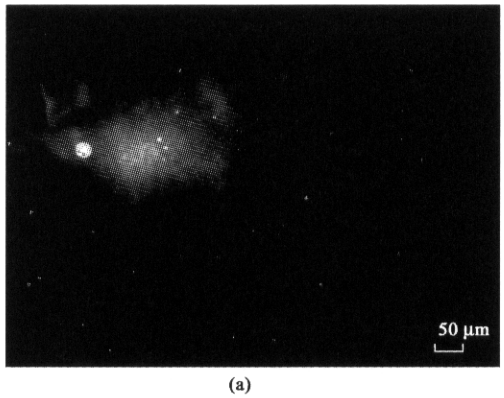


Fig.3 Shoot and root POD activity of *Viaia faba*
The same as Fig.1

Typical comet images of DNA damage in the leaves of *Vicia faba* are shown in Fig.4. In the treatments with GM the comet images had smaller comet tails and fainter fluorescent density, which indicates that there was less DNA migration from the nuclei. The tail length, tail DNA, TM value and OTM value were used to quantify DNA damage (Fig.5a, b, c). All these parameters were significantly lower in GM plants compared to NM plants, showing that the colonization of GM decreased the DNA damages induced by the HMs.



(a)

(b)

Fig.4 Comet images formed by DNA migration in electrophoresis field in the cells of *Vicia faba* leaves
a. NM plants; b. GM plants

3 Discussion

Fuyang District environmental problems associated with fast development of township and households during mine exploitation, appearing in China. Large area of arable land is occupied and contaminated with solid wastes and sludge from mine refining containing many kinds of HMs. The farmland has also been contaminated by irrigation with the wastewater from the factory. This mode of irrigation causes deterioration in soil quality.

It has been suggested that mycorrhizas are beneficial to plant nutrient uptake especially to P uptake (Smith and Read, 1997; Jayachandran and

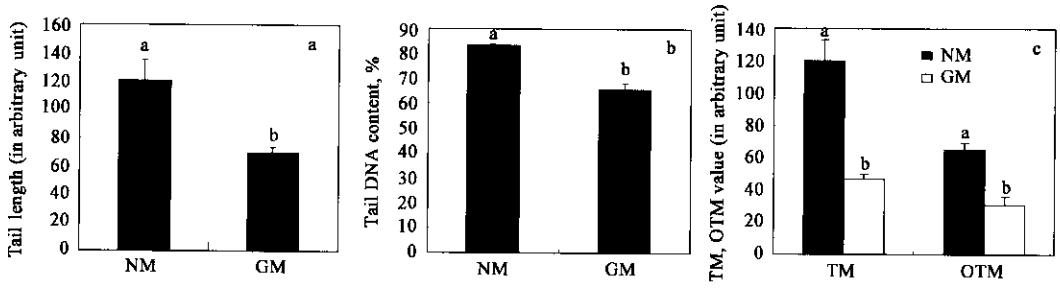


Fig.5 DNA damages in the leaves of *Vicia faba* induced by heavy metals in soil under different treatments
a. tail length; b. tail DNA content; c. TM and OTM value; the same as Fig.1

Shetty, 2003; Cavagnaro *et al.*, 2004; Burleigh *et al.*, 2002) and tolerance to heavy metals (Zhang *et al.*, 2005; Chen *et al.*, 2004); and that AMF might assist pioneering plant species to colonize metal contaminated sites (Vogel-Mikuš *et al.*, 2005; Khan *et al.*, 2000). In the present study, *Vicia faba* inoculated by GM was well colonized and GM plants were larger and had better tolerance to HMs. Better mineral nutrition such as P uptake and lower heavy metal uptake and transport may be the possible mechanisms for success of mycorrhizal plants growing in soils with excessive levels of metals (Liao *et al.*, 2003; Weissenhorn *et al.*, 1995, Zhang *et al.*, 2005). That more P was taken up by GM plants both stimulated growth and increased the tolerance of plants to HMs by "dilution effect". No significant increase in HM concentrations was found in the GM treatments except for Cu and Zn concentrations in roots, which might due to the result of dilution effects of better growth induced by AMF.

Besides the dilution effect, mycorrhiza probably increased the tolerance of plants to HMs by other mechanisms, such as HM immobilization by AM roots compared with NM roots. It has been reported that Pb was unevenly distributed in roots, where different root tissues acted as barriers for Pb to entry apoplastic and symplastic and hence reduce Pb transport to the shoot (Trivedi and Erdei, 1992). It was shown in the present study that the values of *R/S* of Pb and Cd were significantly higher in GM plants than in NM plants indicating that GM treatment immobilized HMs in special parts of the roots or in the hyphal structure. According to Andrade *et al.* (2002), acidic polysaccharides found in the cell wall of brown algae have been associated with the capacity of these organisms to accumulated HMs when exposed to high concentration of HMs. AMF might change the components of root cell walls, so as to affect adsorption characteristics, as found have an increase concentrations of HMs in cell walls. Another explanation relates to stucture of AMF in the roots. According to Joner *et al.* (2000), AM fungal structures had a higher capacity for metal binding than other organisms and could induce more HM immobilization in roots and the symbiotic structures. Therefore, the compartmentation of HMs in the roots would alleviate the toxicity induced by a mixture of HMs, which is similar to the results of the present study.

When HMs enter the protoplast of plants with high concentrations, they could disturb the normal metabolism. One of the mechanisms of HMs toxicity is considered to induce the production of reactive oxygen species (ROS) and may result in significant damage to cellular constituents (Sinha *et al.*, 2005) These ROS include superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), which are produced as by-products during membrane linked electron transport activities as well as by a number of metabolic pathways (Shah *et al.*, 2001). ROS could cause damages to bio-molecules such as membrane lipids, proteins, chloroplast pigments, enzymes and nucleic acids (Mishra and Singhal, 1992). Plants are protected against oxidative stress by the operation of intricate antioxidative systems, comprising both enzymatic systems such as superoxide dismutase (SOD), peroxidases (POD), catalase (CAT), and non-enzymic systems, that acts as free radical scavengers, such as ascorbic acid, thiols, soluble protein and GSH (Sinha *et al.*, 2005). Soluble protein could contribute to oxidative damage in plants by affecting the cell potential energy of infiltration (Zhang *et al.*, 1999). Higher soluble protein content indicated higher stress in the plants. In the present study, soluble protein in the roots were decreased by GM inoculation compared to NM plants, which suggests that *Glomus mosseae* may alleviate the oxidative stress induced by HMs mixture in the roots which maybe lied in the increased immobilization of HMs in root cell wall of hyphe structure. Plant POD are considered to play a role in the antioxidant defense by converting H_2O_2 to H_2O (Sudhakar *et al.*, 2001) and the activity of POD could be used as a parameter of oxidative stress induced by heavy metals. In the present study, inoculation with *Glomus mosseae* decreased POD activity, suggesting a lower oxidative stress in the roots of GM plants and better growth. The better growth was also reflected by the lower DNA damage in the AM plants induced by mixed metal contamination compared to the NM plants. Many previous studies have shown that Cd is a genotoxic

pollutant and that it can induce DNA damage directly or indirectly (Toppi and Gabbrielli, 1999; Pennec and Pennec, 2001, Rucinska *et al.*, 2004, Lin *et al.*, 2005a, b; Wyszko, 2003, Gichner, 2003). However, Cd in shoots of GM and NM plants had no significant difference, which might not be an explanation for lower DNA damage in GM plants. In contrast, that Pb concentration in shoots of GM plants was lower than that in NM plants might contribute to lower DNA damage in GM plants in present study.

4 Conclusions

The formation of AM symbiosis can play an important role in stimulating the growth of *Vicia faba* by some mechanisms that include improved nutrition, HMs immobilization in roots or the fungal structure, oxidative stress alleviation and lower DNA damage. This suggests that AMF could be used in revegetation on a contaminated soil.

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