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Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal-contaminated field site

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Using transgenic plants and plant growth-promoting bacteria as phytoremediation methods increased plant tolerance at a metal-contaminated field site under low flood conditions.

Abstract

The growth of transgenic canola (*Brassica napus*) expressing a gene for the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase was compared to non-transformed canola exposed to flooding and elevated soil Ni concentration, in situ. In addition, the ability of the plant growth-promoting bacterium *Pseudomonas putida* UW4, which also expresses ACC deaminase, to facilitate the growth of non-transformed and transgenic canola under the above mentioned conditions was examined. Transgenic canola and/or canola treated with *P. putida* UW4 had greater shoot biomass compared to non-transformed canola under low flood-stress conditions. Under high flood-stress conditions, shoot biomass was reduced and Ni accumulation was increased in all instances relative to low flood-stress conditions. This is the first field study to document the increase in plant tolerance utilizing transgenic plants and plant growth-promoting bacteria exposed to multiple stressors.

Keywords: Transgenic canola; Plant growth-promoting bacteria; 1-Aminocyclopropane-1-carboxylate deaminase; Nickel; Flooding

1. Introduction

Reducing the effects of environmental stressors on plant growth is advantageous in agriculture and horticulture as well as in more newly developed areas of environmental management such as phytoremediation of contaminated soils. For metal phytoremediation purposes, transgenic plants may be manufactured to synthesize a product that alters metal detoxification or accumulation (Krämer and Chardonnens, 2001; Clemens et al., 2002), or decreases ethylene synthesis to reduce the deleterious plant response to metal stress (Stearns and Glick, 2003). While there are several transgene products that can alter ethylene synthesis, laboratory studies of transgenic plants expressing 1-aminocyclopropane-1-carboxylate (ACC) deaminase have shown increased resistance to a variety of environmental stressors including flooding (Grichko and Glick, 2001a), phytopathogens (Robison et al., 2001), salt (Sergeeva et al., 2006) and metal contamination (Stearns et al., 2005; Grichko et al., 2000; Nie et al., 2002). The enzyme ACC deaminase catalyzes the breakdown of ACC to α-ketobutyrate and ammonia, in turn reducing the quantity of ACC available for oxidation to ethylene. The activity of

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ACC deaminase in transgenic tomato plants has been shown to reduce the effects of flooding stress on growth compared to non-transformed plants (Grichko and Glick, 2001a).

Another approach to reducing the effects of environmental stressors on plant growth involves the use of plant growth-promoting bacteria (Glick, 1995). The enhancement of crop plant growth using plant growth-promoting bacteria is well documented (Reed and Glick, 2004) and more recently these organisms have been used to reduce plant stress associated with phytoremediation strategies for metal and polycyclic aromatic hydrocarbon (PAH) contaminated soils (Reed and Glick, 2005). Plant growth-promoting bacteria expressing ACC deaminase increase plant tolerance to phytopathogens (Wang et al., 2000); flooding (Grichko and Glick, 2001b); salt (Mayak et al., 2004a); water deprivation (Mayak et al., 2004b); and contaminants such as creosote (Reed and Glick, 2005), copper (Reed and Glick, 2005; Reed et al., 2005), cadmium (Belimov et al., 2005) and nickel (Burd et al., 1998). Plant growth-promoting bacteria are typically more effective at protecting plants against the deleterious effects of various environmental stresses than are ACC deaminase transgenic plants. This is likely due to the fact that plant growth-promoting bacteria promote plant growth by several mechanisms, including the synthesis of indoleacetic acid (IAA) and siderophores, and not just by lowering plant ethylene levels.

While there has been considerable laboratory research on transgenic plants and plant growth-promoting bacteria for use in phytoremediation strategies, there are few examples of the use of this technology under field conditions. The objectives of the present study were to determine, in the field, whether transgenic canola plants expressing ACC deaminase activity under the transcriptional control of the root specific *rolD* promoter could reduce the stress associated with metal exposure and flooding relative to non-transformed canola, and whether the addition of the plant growth-promoting bacterium *Pseudomonas putida* UW4 which expresses ACC deaminase and produces IAA, could facilitate plant growth under these conditions.

2. Materials and methods

2.1. Bacterial strain

P. putida UW4, from a rhizosphere soil sample associated with reeds, was isolated based on the ability to utilize ACC as a sole source of nitrogen (Glick et al., 1995; Shah et al., 1997) and previously identified as *Enterobacter cloacae* UW4 (Shah et al., 1997) or *Pseudomonas* sp. UW4 (Glick et al., 1995). Based on the results obtained using a Biolog Kit (MicroLog System, Release 4.0) and the 16s rDNA sequence of the bacterium (AYSS9493), this strain is *P. putida* Biotype A (Hontzeas et al., 2005).

P. putida UW4 was grown in tryptic soybean broth, centrifuged, the pellet washed twice with 30 mM $MgSO_4$ and resuspended in sterile deionized water. The final formulation utilized a bacterial suspension of $OD_{600} = 0.5$ (approximately 3×10^8 colony forming units/ml). The bacterial suspension of *P. putida* UW4 was prepared at the University of Waterloo, placed in sterile plastic screw cap 500 ml bottles and stored on ice for immediate transport to the field plot. At the field plot, two ml of the bacterial suspension was applied per seed hole and the surrounding area (4 cm diameter), where applicable.

2.2. Plants

The plants used in this study were non-transformed and double-copy transgenic canola (*Brassica napus* L. cv. Westar). For the transgenic canola (Stearns et al., 2005), the gene for ACC deaminase was from *P. putida* UW4 (Shah et al., 1998). The canola cultivar contained the ACC deaminase gene under the control of the *rolD* promoter from *Agrobacterium rhizogenes* which is a root specific plant promoter in tobacco (Elmayan and Tepfer, 1995). Three month old seeds were provided by S. Shah (Alberta Research Council, Vegreville, Alberta, Canada).

2.3. Field site

The field site consisted of a 625 m^2 area in Port Colborne, Ontario, Canada. As a result of historical aerial deposition from a local nickel refinery, the soil in the area is contaminated with Ni and Cu (McIlveen and Negusanti, 1994).

2.4. Planting and harvesting methods

In preparation for planting, 1.5% Round-up® (Monsanto Canada, Winnipeg, MB, CA) was applied approximately 2 weeks prior to planting. A week following the herbicide application, the area was ploughed and raked, the plot (18 m × 6 m) established and areas between subplots (60 cm) were covered with landscape fabric to reduce weed growth. Prior to planting, soil samples for chemical analyses were collected in triplicate at a depth of 3 to 5 cm from each subplot. The plot consisted of 16 subplots (1 m × 4 m) with 4 subplots per treatment arranged in a Latin square. The 4 treatments consisted of non-transformed canola (*B. napus* L., cv. Westar) (referred to as the control) (C), ACC deaminase-containing transgenic canola (T), C inoculated with *P. putida* UW4 (C + UW4), and T inoculated with *P. putida* UW4 (T + UW4). Within each subplot there were 4 rows of 25 seeds planted at a depth of 1 to 2 cm with 20 cm between rows and 15 cm between seeds. A total of 400 seeds per treatment were planted.

Seeds were planted August 31 or September 1, 2004 and plants harvested prior to flowering, on October 4 or 5, 2004, respectively (50% per treatment per day). The field plot was examined weekly to assess germination and survival, and to maintain the crop (watering and weeding) as required. During the 34-day (Aug. 31–Oct. 4; Sept. 1–Oct. 5) growing period, daily temperatures ranged from 8.3 to 24.3 °C with an average of 18.2 °C (Environment Canada, 2005). On September 8, precipitation was 96 mm accounting for 79% of the total precipitation (121.2 mm) for the growing period. As a result of this precipitation the canola field plot was severely flooded. Plants were submerged for a minimum of 1–2 days and the soil remained saturated for a minimum of 12 days after the rain. Due to the slope of the land, there was an uneven distribution of water among subplots. Therefore, to assess the effect of flooding on canola growth, each subplot was classified as high (+++), intermediate (++), or low (+) flood stress based on the level of flooding and % seedling survival per subplot at the time of harvest. Subplots with high flood stress (+++) had a high level of flooding and <50% plant survival. Subplots with low flood stress (+) had a low level of flooding and ≥50% plant survival. Subplots with intermediate flood stress (++) had a high level of flooding but ≥50% plant survival.

At harvest, individual plants (shoots and roots) were carefully removed from the soil, placed in labelled plastic bags and stored on ice for transport to the University of Waterloo for measurements of shoot length (cm) and dry weight (g). Shoots and roots were initially cleaned with tap water then rinsed in 10 mM EDTA (roots only) followed by deionized water, dried at 80 °C for 2 days and weighed. Sub-samples of cleaned, dried shoots and roots were stored in plastic vials for metal analysis.

2.5. Chemical analyses

Soil samples collected from subplots within the canola field plot (August 31, 2004) were analyzed at the University of Guelph Laboratory Services Division (Guelph, Ontario, Canada) for nutrient and metal content. For metal

analyses, soil samples were digested with concentrated nitric acid and hydrochloric acid and measured using the Varian Vista Pro (radial) inductively coupled plasma-optical emission spectrometer (ICP-OES) (Varian Canada, Inc., Mississauga, ON, CA). Mercury was measured using a cold vapour atomic absorption spectrophotometer (Varian SpectrAA 220 FS) (Varian Canada, Inc., Mississauga, ON, CA). For total and inorganic carbon, soil samples were combusted at 1475 °C and 475 °C, respectively. The quantity of organic carbon in the soil was calculated from the difference between total carbon content and inorganic carbon content. Organic matter content of the soil was determined using the Walkley-Black method. Plant available Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} were determined using ammonium acetate extraction and P using sodium bicarbonate extraction. Total nitrogen was determined using the Dumas method on a Leco instrument. Soil NH_4, NO_3^{2-} and NO_2^{-} concentrations were determined using a KCl extraction method. Soil pH was determined using a saturated paste made with deionized water.

For plant tissue metal analyses, whole dried shoots or roots were finely ground and stored in 15 ml plastic scintillation vials. Approximately 30 mg of plant tissue samples were placed in 1.5 ml microcentrifuge tubes (Progene, St-Laurent, QC, CA), in duplicate, for each plant. To digest the tissue, 0.75 ml of 1 N HNO_3 (Fisher Scientific, Nepean, ON, CA) was added to each tube which was then vortexed and incubated at 65 °C for 3 h. Tubes were allowed to cool, centrifuged at 20 000 × *g* for 10 min and then 100 µl of the supernatant was transferred to 900 µl of Milli-Q water in 1.5 ml microcentrifuge tubes and stored at 4 °C prior to analysis. Samples were run on a Varian SpectrAA 880 spectrophotometer, with a GTA 100 graphite furnace (Varian Canada, Inc., Mississauga, ON, CA) (standard curve was between 1 and 5 µM Ni).

2.6. Statistics

Data for all parameters were expressed as the mean ± standard error (SE) for each subplot or treatment. Significant differences between the four treatments under low and high flood stress were determined using analysis of variance followed by a Tukey test. All statistical analyses were conducted at $\alpha = 0.05$ using SYSTAT 10 (Statistical Package for the Social Sciences, 2000).

3. Results

At the field site, elevated soil levels of Ni and Cu as well as other metals and major ions were common (Table 1). The organic soil consisted of 48.2% clay, 42.2% silt and 9.6% sand; it contained 50–52% dry weight organic matter. Inorganic and organic carbon ranged from 0.55–0.7% dry weight and 35–40.3% dry weight, respectively. Further details of soil chemistry are provided in Table 1.

At the time of the flood the majority of the canola seeds had germinated although assessing germination was complicated by the excess water in some plots which may have accounted for the high variation (51% to 96% germination) between the 16 subplots (Table 2). Plant survival at harvest time was reduced and also varied between subplots (3% to 93%) (Table 2). Under low flood stress, germination for non-transformed and transgenic treatments was 85–96% and 72–88%, respectively. Flood stress was assessed for all subplot however due to the low number of subplot classified as intermediate (++), these plots could not be used to evaluate differences between the canola treatments and flood stress (Table 2).

During the 34-day growing period, there were noticeable differences in the growth of canola as a function of flood-stress conditions. At harvest, canola shoot length and biomass (dry weight) were reduced for all treatments in the high vs. low flood-stress conditions (Fig. 1a,b). Canola plants under high flood-stress conditions had similar growth among the treatments, based on shoot length and biomass, with the exception of C which had significantly greater growth than T and T + UW4 ($p \leq 0.033$). In comparison, plants under low flood stress had significant differences ($p < 0.001$) in growth among

Table 2
Canola germination (%) and survival (%) for the four treatments (non-transformed canola, *B. napus* L., cv. Westar (C); transgenic canola (T); C inoculated with *P. putida* UW4 (C + UW4); T inoculated with *P. putida* UW4 (T + UW4)) under varying flood-stress conditions (high, +++; intermediate, ++; low, +)

Treatment	Plot #	Level of Flooding[a]	Germination (%)[b]	Survival (%)[c]	Flood Stress[d]
C	1	high	84	46	+++
	8	high	79	46	+++
	11	low	85	74	+
	14	high	72	68	++
C + UW4	2	high	71	18	+++
	5	high	81	78	++
	12	low	86	80	+
	15	low	96	93	+
T	3	high	74	23	+++
	6	high	67	16	+++
	9	low	79	79	+
	16	low	72	68	+
T + UW4	4	high	51	3	+++
	7	high	80	68	++
	10	high	75	39	+++
	13	low	88	84	+

[a] Level of flooding was assessed one day following precipitation.
[b] Germination estimated on September 14, 2004.
[c] Calculated from number of plants at harvest (Oct. 4 and 5).
[d] Classification of flood stress based on level of flooding and % survival.

Table 1
Chemistry and metal concentrations in soil samples collected from subplots within the canola field plot (August 31, 2004)

Soil Chemistry										
pH	Ca^{2+}[a]	K^{+}[a]	Mg^{2+}[a]	Na^{+}[a]	TN[b]	NH_4	NO_2^{-}	NO_3^{2-}	P[a]	
6.1	6601	343	722	27	2.44	11.5	<0.1	<0.1	86	
Metal Concentrations[c]										
As	Cd	Co	Cr	Cu	Hg	Mo	Ni	Pb	Se	Zn
18 ± 1	<1.0	38 ± 1	13.8 ± 0.9	390 ± 7	0.20 ± 0.00	<2.5	2983 ± 79	36 ± 1	6.3 ± 0.1	115 ± 3

Measurements are in µg/g soil unless otherwise stated.
[a] Plant available measurements in mg/L soil (equivalent to µg/g soil).
[b] % dry weight.
[c] Total metal concentration reported as mean ± SE with the exception of Cd and Mo.

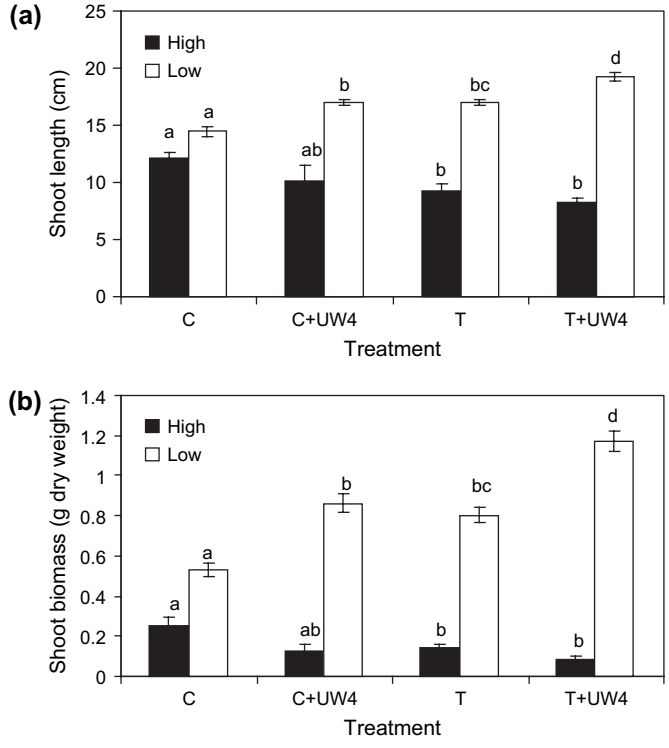

Fig. 1. Mean ± SE shoot length (cm) (a); and shoot biomass (g dry weight) (b) for canola from low and high flood-stress conditions. Treatments include non-transformed canola *B. napus* L., cv. Westar (C); transgenic canola (T); C inoculated with *P. putida* UW4 (C + UW4); T inoculated with *P. putida* UW4 (T + UW4)). Sample numbers (*n*) range from 18 to 171 plants for shoot length and 18–84 plants for shoot biomass. Different letters represent statistical differences between treatments for low ($p < 0.001$) and high ($p < 0.05$) flood-stress conditions.

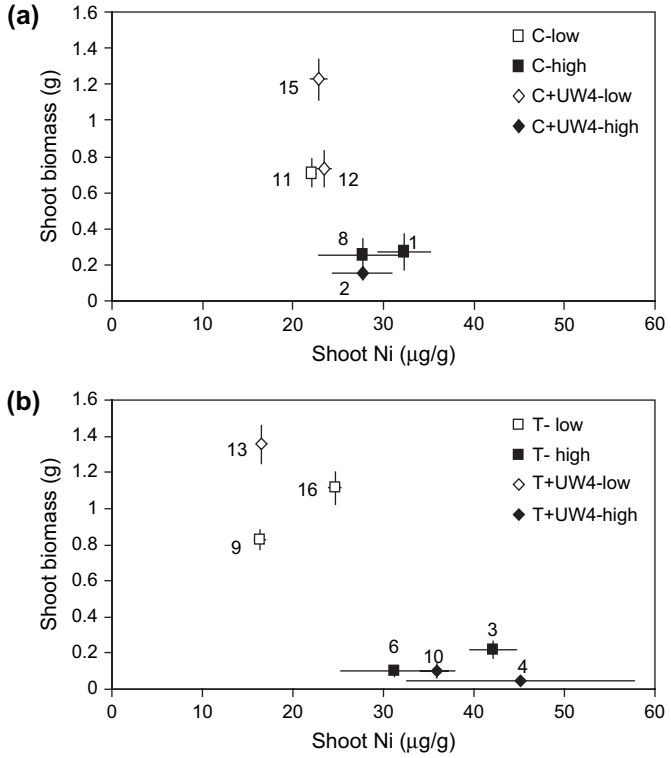

Fig. 2. Mean ± SE shoot biomass (g dry weight) vs. shoot Ni concentration (μg/g dry weight) for: (a) non-transformed canola (*B. napus* L., cv. Westar) (C) and C inoculated with *P. putida* UW4 (C + UW4); (b) transgenic canola (T) and T inoculated with *P. putida* UW4 (T + UW4). Closed symbols represent high flood-stress conditions and open symbols represent low flood-stress conditions. Numbers beside the symbol refer to the subplot #.

all treatments with the exception of C + UW4 and T. Under low flood stress, transgenic canola had increased growth (34%) relative to C canola, and the addition of *P. putida* UW4 enhanced the growth of both C (38%) and T canola (31%).

To better understand the potential impact of Ni contamination on canola growth under flood-stress conditions, Ni concentrations were measured in canola shoots and roots. Concentrations of Ni in the shoots and roots were compared for subplots under low flood-stress conditions with soil concentrations ranging from 2800–3100 μg Ni/g dry weight of soil. In general, canola roots had higher (>two-fold) concentrations of Ni relative to shoots. For example, C had shoot and root Ni concentrations of 20.6 ± 0.7 and 56.7 ± 2.6 (μg/g dry weight), respectively. The transfer of Ni from the roots to the shoots was similar among canola treatments with translocation efficiencies ((shoot Ni/root Ni) × 100) of $36.4 \pm 1.2\%$ (C), $39.4 \pm 1.8\%$ (C + UW4), $44.7 \pm 3.4\%$ (T) and $38.8 \pm 1.2\%$ (T + UW4).

Shoot Ni concentrations were used to compare high and low flood-stress conditions. Both non-transformed (Fig. 2a) and transgenic (Fig. 2b) canola showed trends of lower growth and higher Ni concentration for subplots under high flood-stress conditions. Shoot Ni concentrations were more variable within subplots under high flood-stress conditions. Based on data pooled per treatment, transgenic canola treatments had statistically higher shoot Ni concentrations under high vs. low flood-stress conditions ($p < 0.001$) (Table 3). Shoot Ni concentrations were similar among the treatments for low or high flood-stress conditions with the exception of the difference between C + UW4 and T under high flood-stress conditions (Table 3). The addition of *P. putida* UW4 did not affect

Table 3
Mean ± SE (*n*) and range for Ni concentrations in canola shoots for the four treatments (non-transformed canola, *B. napus* L., cv. Westar (C); transgenic canola (T); C inoculated with *P. putida* UW4 (C + UW4); T inoculated with *P. putida* UW4 (T + UW4)) under low or high flood-stress conditions

Treatment	Flood stress	Mean ± SE (*n*) (μg/g dry wt)[x]	Range (μg/g dry wt)
C	high	29.88 ± 2.90 (15)^{a}	0.06–49.05
	low	22.14 ± 0.43 (20)	18.46–24.85
C + UW4	high	27.67 ± 3.28 (14)^{ab}	14.95–50.49
	low	23.17 ± 0.59 (22)	18.04–27.59
T	high	37.13 ± 3.17 (24)^{acA}	16.07–81.41
	low	20.72 ± 1.03 (21)^{B}	14.22–27.88
T + UW4	high	38.01 ± 2.91 (9)^{aA}	30.95–57.78
	low	16.56 ± 0.50 (22)^{B}	8.69–20.59

[x]Different small letters represent statistical differences ($p < 0.05$) between the treatments within low or high flood-stress conditions; different capital letters represent statistical differences ($p < 0.001$) between low or high flood stress for a given treatment.

Ni concentrations in the shoots of canola treatments under low or high flood-stress conditions.

4. Discussion

In the experiment reported here, transgenic canola in the absence and especially in the presence of *P. putida* UW4 had significantly increased growth compared to non-transformed canola in metal-contaminated soil under low flood-stress conditions. Overall the growth of canola in this study was greatly reduced in comparison to the average shoot dry weight (>2.0 g) for transgenic and non-transformed canola grown from seed at the same metal-contaminated field site (June, 2004) in a preliminary study (unpublished data). This suggests that flood stress, evident under high and low flood conditions was likely the major factor that contributed to the reduction of canola growth.

The use of *P. putida* UW4 increased growth in both transgenic and non-transformed canola in metal-contaminated soil under low flood-stress conditions. It was previously demonstrated in laboratory studies that *P. putida* UW4 can lower ethylene levels in tomato plants and increase plant biomass when exposed to environmental stressors such as flooding (Grichko and Glick, 2001b). In addition, the presence of *P. putida* UW4 had no affect on the concentration of Ni in the shoots under high or low flood-stress conditions. Similarly, Burd et al. (1998) found that the bacterium, *K. ascorbata* SUD165 did not affect nickel uptake in canola. Although, there are studies that have shown reduced (Petrisor et al., 2004) or increased metal content in plant tissues following bacterial inoculation (Hoflich and Metz, 1997; de Souza et al., 1999).

In the current study there are two major stressors, flooding and elevated metal content, that influenced the response of canola plants and possibly the uptake of metals. For low flood conditions, all treatments had similar shoot Ni concentrations but non-transformed canola plants had reduced shoot biomass. Shoot Ni concentrations were lower than concentrations (>50 μg Ni/g shoot) causing reduced growth in non-transformed canola in laboratory studies (Stearns et al., 2005) suggesting that flooding stress was the main contributor to reductions in the growth of canola at the metal-contaminated site. However, under high flood-stress conditions, all canola treatments had reduced growth and elevated shoot Ni concentrations but non-transformed canola had significantly greater shoot biomass. Only transgenic plants had shoot Ni concentrations that were significantly higher compared to low flood-stress conditions which may account for the reduced growth of transgenic canola plants compared to the non-transformed canola under high flood-stress conditions.

Soil conditions affecting Ni uptake from roots and accumulation in shoots may have been affected by flooding based on the elevated Ni concentrations in canola shoots exposed to high flood-stress conditions. Species of Ni hyperaccumulators grown in Ni-contaminated soils with 80 and 100% water holding capacity (WHC) were found to have higher foliar concentrations of Ni compared to treatments with lower WHC (Angle et al., 2003). In contrast, flooded rice plants, although generally insensitive to ethylene inhibition due to flooding, had reduced or similar concentrations of shoot Ni compared to non-flooded rice plants held in soil at ~80% WHC (Kashem and Singh, 2001). Variation in Ni accumulation between different soils in the study by Kashem and Singh (2001) indicated that changes in Ni accumulation due to flooding may depend on soil parameters. Also, translocation of Ni from root to shoot has been found to vary among different species depending on soil conditions, for example, soil acidity (Pinel et al., 2003). In the current study, Ni translocation from the root to shoot was between 36 and 45% compared to <20% for transgenic canola in laboratory studies (Stearns et al., 2005) and ~50% for other plant species (Pinel et al., 2003).

5. Conclusions

In summary, ACC deaminase-containing transgenic canola and *P. putida* UW4 increased plant biomass, separately or in combination, under pressure from multiple environmental stressors (flooding and elevated Ni concentration) in this field investigation. Using either transgenic canola or *P. putida* UW4 provided similar enhanced and additive tolerance under low flood-stress conditions. Relative changes in biomass and Ni accumulation for canola treatments were dependant on the severity of the flood conditions. The functioning of transgenic canola and *P. putida* UW4 under field conditions was consistent with laboratory studies that examined the effects of *P. putida* UW4 (Grichko and Glick, 2001b) and transgenic tomato plants (Grichko and Glick, 2001a) on flooding and the effects of transgenic canola exposed to Ni-spiked soil (Stearns et al., 2005).

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