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Effect of nitrate fertilization and saline stress on the contents of active constituents of *Echinacea angustifolia* DC

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Abstract

In this study, the effect of nitrogen form (nitrate and ammonium in the molar ratio of 1:1 or 1:0), supplied to the plant or NaCl salinity (up to a concentration of 50 mol m^{-3}) of the feeding solution, on the content of selected caffeic acid derivatives and the activities of phenylalanine ammonia lyase (PAL; EC. 4.3.1.5) and shikimate dehydrogenase (SKDH; EC 1.1.1.25) were investigated in the leaves and the roots of *Echinacea angustifolia* DC, grown hydroponically. Two experiments were conducted under glasshouse conditions with seedlings transplanted in aerated stagnant nutrient solution (floating raft system) four weeks after emergence. The nitrate/ammonium ratio in the nutrient solution did not affect plant growth or dry matter partitioning, but it influenced the concentration of determined metabolites; in particular, the root concentrations of chlorogenic acid, echinacoside and caffeic acid were significantly higher in the plants grown with nitrate as sole source of nitrogen. NaCl salinity reduced plant growth, by reducing the biomass allocated to the inflorescences, and enhanced the accumulation of chlorogenic acid and cichoric acids, at least in the roots.
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Keywords: *Echinacea angustifolia*; Hydroponics; NO_3^- : NH_4^+ ratio; PAL activity; Saline stress; SKDH activity

1. Introduction

Echinacea spp. (coneflower) is an herb widely used for its medicinal properties, especially in the USA and Europe (Barret, 2003; Li, 1998). The extract of coneflower has significant immunomodulatory (Barret, 2003; Bennett & Wallsgrove, 1994; Goel et al., 2002; Heiser & Elstner, 1998) and antioxidant activities (Hu & Kitts, 2000; Thygesen, Thulin, Mortensen, Skibsted, & Molgaard, 2007). Among the 11 known taxa of *Echinacea*, the three species *E. angustifolia* DC. var. *angustifolia*, *E. pallida* (Nutt.) and *E. purpurea* (L.) Moench are those used for their pharmacological activity (Binns, Baum, & Arnason, 2002). The first two species are grown or collected mostly for the roots while the aerial parts are used in the case of the third species. The most important organic compounds contained in these species responsible for their pharmacological activity are caffeic acid derivatives, alkylamides and polysaccharides (Binns, Baum, et al., 2002; Briskin, 2000; WHO, 1999).

For pharmaceutical use, the plant material must be free of contamination by heavy metals, soil and soil-borne organisms, herbicides and pesticides. In this sense, hydroponics may provide a suitable growing system for the standardized production of high-quality plant material containing active principles, in particular for those medicinal plants, such as *E. angustifolia* DC (purple coneflower), which are cultivated mainly for the roots and are difficult to grow in the open field (Zheng, Dixon, & Saxena, 2006b). In addition, hydroponics also allows regulation of secondary metabolism involved in the accumulation of pharmacological active principles, by opportune management of nutrient solutions.

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Many environmental and genetic factors may affect the levels of the active principles of plant material (e.g. Laasonen et al., 2002) and both biotic and abiotic stress exert a considerable influence on the levels of secondary metabolites in plants, the synthesised metabolites being typically involved in the defence responses of plants (Dixon & Paiva, 1995).

Caffeic acid derivatives are typically synthesised by phenylpropanoid pathway, in which the PAL enzyme plays a key role in diverging primary metabolism from the production of secondary metabolites. For these reasons, it is interesting to study how different growing conditions can improve the accumulation of secondary metabolites.

In this study, the effect of nitrogen form (nitrate and ammonium), supplied to the plant or the NaCl salinity of the nutrient solution, on plant growth and the contents of selected caffeic acid derivatives in leaf and root tissues were investigated in seedlings of *E. angustifolia* DC grown in water culture. At the time this work was initiated, we were not aware of any paper published on the soilless culture of *E. angustifolia* and only recently two works on this subject were published by Zheng, Dixon, Saxena (2006a, 2006b). With respect to the experiments undertaken by Zhen and colleagues, our study considered the activities of some enzymes involved in the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL; EC 4.3.1.5) and shikimate dehydrogenase (SKDH; EC 1.1.1.25), and the possibility of increasing the accumulation of active principles by the application of a moderate salt stress.

2. Materials and methods

2.1. Experimental protocols

The experiments were conducted from February to June 2006 at the University of Pisa (Pisa, Italy) in a glasshouse with minimum (heating) and ventilation air temperatures of 16 and 27 °C, respectively. Maximum photosynthetic photon flux density (PPFD) inside the glasshouse ranged from 500 to 700 μmol m^{-2} s^{-1}.

E. angustifolia seeds (Gold Nugget Seed®, purchased from Jelitto Staudensamen GmbH, Schwarmstedt, Germany) were sown in rockwool tray plugs and, four weeks after emergence, the seedlings were transferred into a floating raft growing system, consisting of polystyrene plug trays floating in plastic tanks with a constant volume (300 l m^{-2}) of stagnant nutrient solution, which was continuously aerated with an air compressor in order to maintain the oxygen content above 6.0 mg l^{-1}. Crop density at transplanting was 122 plants m^{-2} and the survival percentage averaged 80.5 for a density of approximately 98 plants m^{-2} at harvest, which took place 12 (exp. N. 1) or 15 weeks (exp. N. 2) after emergence.

The nutrient solution (NS) was prepared by dissolving appropriate amount of NH_4NO_3, $Ca(NO_3)_2$, $CaCl_2$, KNO_3, K_2SO_4, $K_2H_2PO_4$, $MgSO_4$, NaCl and/or KCl in ground water containing approximately 5.0 mol m^{-3} of NaCl. The compositions were the following: Nutrient Solution 1 (NS1): (NO_3^- : NH_4^+ of 1:0), 8.0 mol m^{-3} N-NO_3^-, 0.5 mol m^{-3} P-$H_2PO_4^-$, 1.0 mol m^{-3} S-SO_4^-, 9.0 mol m^{-3} Cl^-, 5.0 mol m^{-3} K^+, 2.0 mol m^{-3} Ca^{2+}, 1.0 mol m^{-3} Mg^{2+}, 9.0 mol m^{-3} Na^+; Nutrient Solution 2 (NS2): (NO_3^- : NH_4^+ of 1:1), 4.0 mol m^{-3} N-NO_3^-, 4.0 mol m^{-3} N-NH_4^+, 0.5 mol m^{-3} P-$H_2PO_4^-$, 3.0 mol m^{-3} S-$SO_4{}^-$, 12.0 mol m^{-3} Cl^-, 5.0 mol m^{-3} K^+, 2.0 mol m^{-3} Ca^{2+}, 1.0 mol m^{-3} Mg^{2+}, 8.0 mol m^{-3} Na^+. In experiment N. 2, one group of plants was grown in nutrient solution enriched with NaCl up to a concentration of 50 mol m^{-3}. Trace elements were added as EDTA chelates. In all cases, the nutrient solution of each tank was replaced completely every four weeks. The electrical conductivity (EC) oscillated between 1.5 and 2.0 dS m^{-1} in non-salinized nutrient solution and reached 7.5 dS m^{-1} in NaCl salinity treatment. The pH was maintained between 5.5 and 7.0 by frequent adjustment with sulphuric acid.

2.2. Plant determinations

In each experiment, six samples, consisting of one plant, were randomly collected from hydroponics systems. The plants were washed in tap water, rapidly rinsed in deionised water, dried with a towel and then separated into roots, leaves and inflorescences.

The extraction of active metabolites was performed on samples previously dried at 50 °C in a ventilated oven. This temperature was chosen since it resembles the conditions used by *Echinacea* growers or collectors. Dried samples were maintained in desiccators until they were analysed for selected metabolites, generally not later than a couple of weeks after sampling. Dry weight was determined in aliquots of each sample by desiccation at 80 °C until a constant weight was reached.

For the determination of caffeic acid derivatives, the protocol reported by Luo, Chen, Yao, and Zeng (2003) was applied, with minor modifications. Briefly, 0.2 g of dried tissue was ground in a mortar. The powder was mixed with 10 ml of extraction solvent (MeOH:H_2O 70:30 v/v) and shaken for 4 h in an orbital shaker on an ice bath. The extracted samples were maintained for three days at −20 °C and subsequently centrifuged for 2 min at 5000 rpm. The supernatant was filtered through a 2.5 cm diameter, 0.45 μm PTFE membrane syringe filter, and injected for HPLC analysis.

HPLC grade solvents and the following chemically pure standards were used: echinacoside, cynarine, caftaric acid, cichoric acid (Phytolab GmbH, Vestenbergsgreuth, Germany); chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid (Sigma–Aldrich, Milano, Italy). HPLC analytical equipment was composed of a Jasco PU-2089 four-solvent low-pressure gradient pump and an UV-2077 UV/Vis multichannel detector. Analyses were performed using a Macherey–Nagel 250/4.6 Nucleosil® 100–5 C_{18} column equipped with a guard column, with a gradient of two solvents: (A) acetonitrile; (B) 0.1% aqueous solution of

o-phosphoric acid. The gradient elution was programmed as follows: 0.0–0.4 min, B 95%; 0.4–0.5 min, B 95–85%; 0.5–10 min, B 85–80%; 10–20 min, B 80–60%; 20–21 min, B 60–5%; 21–25 min, B 5%; 25–26 min, B 5–95%; 26–30 min, B 95%. Other chromatographic conditions were as follows: flow rate 1 ml min^{-1}, detection wavelength 325 nm, injection volume 20 μl, room temperature 23–29 °C. Chromatograms were also recorded at 280, 300 and 350 nm. Caffeic acid derivatives were identified through their retention time, by comparison with those of reference standards, and quantified on the basis of the integrated peak area, as compared with a standard curve.

PAL activity was measured as previously described by Guidi, Degl'Innocenti, Genovesi, and Soldatini (2005), with some modifications. Two grams of tissue were homogenized with 16 ml of 50 mM borate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol (β-Mesh) and 0.2 g polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000*g* at 4 °C for 20 min. The supernatant was assayed for PAL activity after the addition of 0.55 ml of 50 mM L-phenylalanine and incubated at 40 °C for 30 min. The absorbance was measured at 290 nm before and after incubation. PAL activity was expressed as μmol *trans*-cinnamic acid min^{-1} mg^{-1} prot.

For the enzymatic assay of SKDH, a sample (0.2–0.4 g) of fresh tissue was homogenized in the presence of 0.1 M K-phosphate buffer, pH 7.4, containing 0.5 mM dithiothreitol (DTT), 2 mM L-cysteine, 2 mM ethylenediaminetetracetic acid, 8 mM β-Mesh and 0.5 g of PVPP. The spectrophotometric assay for SKDH was performed at 25 °C in a reaction medium containing 4 mM shikimic acid and 2 mM $NADP^+$ in 0.1 M Tris [tris-(hydroxymethyl)aminomethane]–HCl buffer, pH 9. The reaction was initiated by adding enzymatic extract and, following the NADP reduction at 340 nm for 5 min, SKDH activity was expressed as μmol NADPH min^{-1} mg^{-1} prot.

Protein determinations were performed according to the method of Lowry, Roxebrough, Farr, and Randall (1951).

2.3. Statistical analysis

Metabolite concentrations were subjected to a two-way analysis of variance (ANOVA) and the means were separated with the least significant differences (LSD) test for $P = 0.05$. The Student *t* test was applied for data of growth and enzyme activities.

3. Results and discussion

The $NO_3^-:NH_4^+$ ratio did not significantly influence the biomass accumulation or its partitioning in the roots and leaves (Table 1), as was also found by Zheng et al. (2006a). As far as the concentration of caffeic acid derivatives is concerned, only four out of eight external standards were detected by HPLC in sampled plants: chlorogenic acid, echinacoside, cynarin and cichoric acid. Their concentrations are listed in Table 2; the other compounds were not revealed or were identified at concentrations below the detection limit (0.005%, on a dry weight basis). There were no differences between the two groups of plants fed with different $NO_3^-:NH_4^+$ ratios as far as the leaf concentration of active metabolites is concerned (Table 2). Conversely, the root concentration was significantly increased in the plants fed with nitrate as a sole source of nitrogen. Overall, root tissues showed much higher contents of chlorogenic acid, echinacoside, cynarin and cichoric acid than did the leaves. Cynarin and cichoric acid were the phenols in root of *E. angustifolia* at the highest concentration.

The activities of some enzymes involved in the metabolism of phenylpropanoid metabolites and determined in leaf and root tissues are shown in Fig. 1. In general, the activities of SKDH and PAL in leaves were significantly higher than those recorded in the roots ($P > 0.01$). No difference was found between the two nitrogen nutrition

Table 1
Growth parameters determined in seedlings of *Echinacea angustifolia* grown hydroponically in a glasshouse using nutrient solutions differing in their $NO_3^-:NH_4^+$ ratios

	$NO_3^-:NH_4^+ = 1:0$	$NO_3^-:NH_4^+ = 1:1$	Significance
Leaf dry weight (g)	0.64 ± 0.01	0.65 ± 0.01	NS
Root dry weight (g)	0.32 ± 0.03	0.31 ± 0.03	NS
Total dry weight (g)	0.96 ± 0.04	0.96 ± 0.04	NS
Root:shoot ratio	0.52 ± 0.04	0.61 ± 0.06	NS

Mean values ± s.e. of six replicates, each replicate consisting of one plant. In the last column the significance of the difference between the two mean is reported. NS: not significant.

Table 2
Root and leaf content of caffeic acid derivatives (μg g^{-1} DW) determined in seedlings of *Echinacea angustifolia* grown hydroponically in a glasshouse using nutrient solutions differing in their $NO_3^-:NH_4^+$ ratios

	$NO_3^-:NH_4^+ = 1:0$	$NO_3^-:NH_4^+ = 1:1$
Chlorogenic acid		
Leaves	75 (15)b	242 (131)b
Roots	908 (295)a	254 (47)b
Echinacoside		
Leaves	530 (111)b	453 (109)b
Roots	844 (88)a	466 (128)b
Cynarin		
Leaves	124 (24)c	191 (40)c
Roots	926 (280)a	560 (219)b
Cichoric acid		
Leaves	243 (23)c	367 (196)bc
Roots	1330 (395)a	582 (253)b
Total detected phenols		
Leaves	972 (91)c	1253 (161)b
Roots	4008 (515)a	1862 (272)b

Mean values (± s.e.) of six replicates, each replicate consisting of one plant. The means flanked by the same letter are not significantly different at 5% level following two-way ANOVA and LSD test.

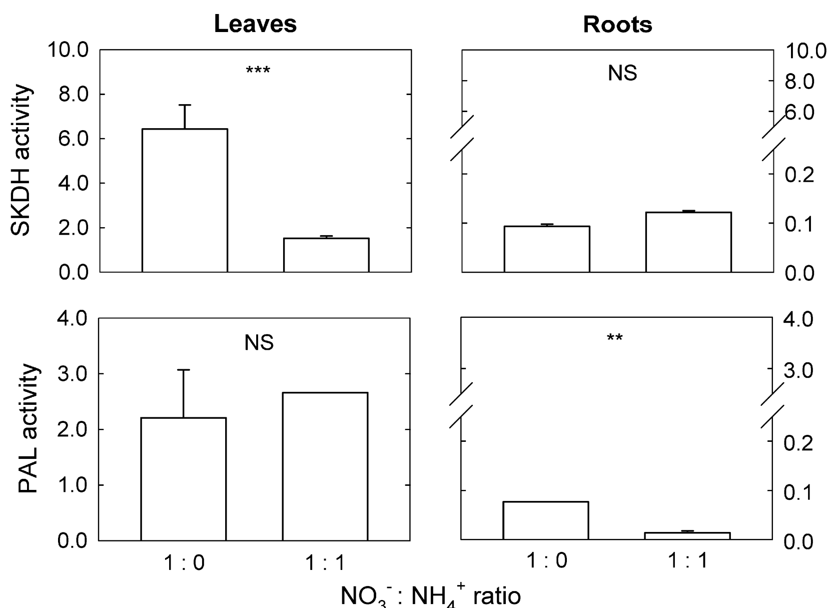

Fig. 1. Activities of shikimate dehydrogenase (SKDH; μmol NADPH min^{-1} mg^{-1} protein) and phenylalanine ammonia lyase (PAL; μmol *trans*-cinammic min^{-1} mg^{-1} protein) determined in leaves and roots of *Echinacea angustifolia* seedlings grown hydroponically in a glasshouse using nutrient solutions differing in their NO_3^- : NH_4^+ ratios. Mean values (± s.e.) of three replicates, each replicate consisting of one plant. For leaves and roots, the significance of the difference between plants grown with different forms of nitrogen is reported (NS: not significant; **: significant at 1% level; ***: significant at 1‰ level).

regimes in the activity of SKDH determined in the roots, while it was significantly higher in the leaves sampled from the plants grown without ammonium in the nutrient solution. Different results were found for PAL activity, which was not influenced by nitrogen solution in leaves while it was markedly reduced in the roots of plants grown with a NO_3^- : NH_4^+ ratio of 1:1.

NaCl salinity reduced total plant biomass in consideration of a lower dry weight of the inflorescences (Table 3); the salinization of the nutrient solution also resulted in an increase of the root:shoot ratio. As with the results of the first experiments, the difference in the salinity of the nutrient solution did not change the leaf concentration of determined metabolites, but it significantly enhanced the root contents of chlorogenic acid, cynarin and cichoric acid (Table 4). When the concentration of total phenols was expressed per plant, a significant reduction was observed; in fact, the total detected phenols decreased from 88.7 to 56.1 μg $plant^{-1}$.

No effect of salinity was observed in the activities of SKDH determined in the leaves and roots sampled from tissues (Fig. 2); by contrast, the activity of PAL increased significantly in the roots of salt-treated plants. Although no measurements indicative of oxidative load were determined in saline stress plants, the variations seen in the con-

Table 3
Growth parameters determined in seedlings of *Echinacea angustifolia* grown hydroponically in a glasshouse using nutrient solutions differing in their NaCl concentrations

	5.0 mol m^{-3} NaCl	50.0 mol m^{-3} NaCl	Significance
Leaf dry weight (g)	1.47 ± 0.46	1.04 ± 0.17	NS
Inflorescence dry weight (g)	10.13 ± 2.15	4.39 ± 1.41	**
Root dry weight (g)	1.53 ± 0.71	1.04 ± 0.20	NS
Total dry weight (g)	13.13 ± 2.77	6.47 ± 1.42	**
Root:shoot ratio	0.13 ± 0.02	0.19 ± 0.06	*

Mean values ± s.e. of six replicates, each replicate consisting of one plant. The significance of the difference between the two means is shown in the last column. NS: not significant; *: significant at 5% level; **: significant at 1% level.

Table 4
Root and leaf contents of caffeic acid derivatives (μg g^{-1} DW) determined in seedlings of *Echinacea angustifolia* grown hydroponically in a glasshouse using nutrient solutions differing in their NaCl concentrations

	5.0 mol m^{-3} NaCl	50.0 mol m^{-3} NaCl
Chlorogenic acid		
Leaves	128 (49)c	nd
Roots	438 (97)b	1085 (227)a
Echinacoside		
Leaves	1695 (157)a	2511 (518)a
Roots	2173 (628)a	2453 (697)a
Cynarin		
Leaves	nd	nd
Roots	990 (314)a	626 (196)b
Cichoric acid		
Leaves	304 (59)bc	109 (23)c
Roots	1028 (591)b	1895 (262)a
Total detected phenols		
Leaves	2127 (206)c	2620 (942)c
Roots	4629 (1187)b	6059 (928)

Mean values (± s.e.) of six replicates, ech replicate consisting of one plant. The means flanked by the same letter are not significantly different at 5% level following two-way ANOVA and LSD test. nd: not detected (the values were below the detection limits).

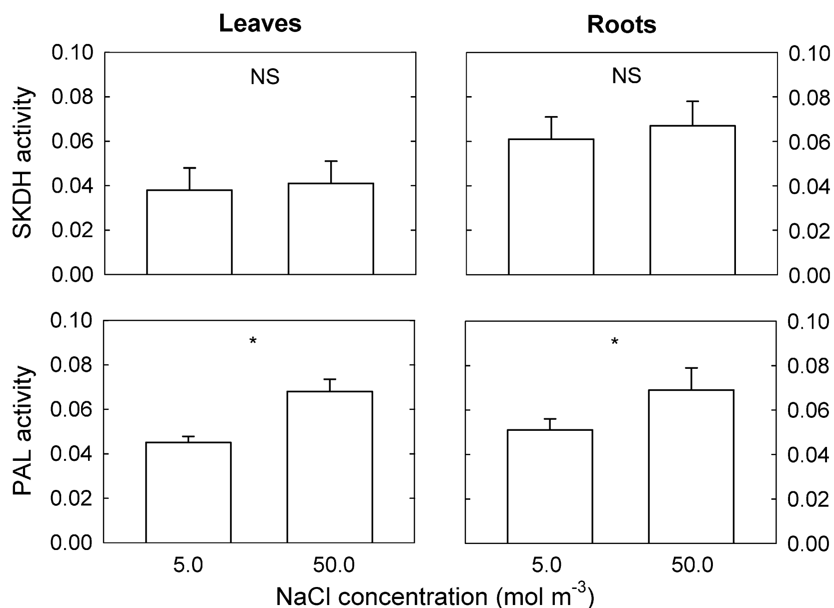

Fig. 2. Activities of shikimate dehydrogenase (SKDH; μmol NADPH min^{-1} mg^{-1} protein) and phenylalanine ammonia lyase (PAL; μmol *trans*-cinammic min^{-1} mg^{-1} protein) determined in leaves and roots of *Echinacea angustifolia* seedlings grown hydroponically in a glasshouse using nutrient solutions differing in NaCl concentration. Mean values (± s.e.) of three replicates, each replicate consisting of one plant. For leaves and roots, the significance of the difference between plants grown with different forms of nitrogen is reported (NS: not significant; *: significant at 5% level).

tent of phenolic compounds could be partly related to the presence of reactive oxygen species (ROS) generated by oxidative stress. Evidence that phenols may confer protection against ROS has been widely obtained (Bennett & Wallsgrove, 1994; Heiser & Elstner, 1998).

Many phenolic compounds in plants are synthesised by the phenylpropanoid pathway which is typically initiated by PAL activity and the identified caffeic acid derivatives (chlorogenic acid, echinacoside cynarine and cichoric acid) in *E. angustifolia* leaf and root tissues were also biosynthesised through this pathway (Shirley, 2001). As shown in Fig. 1, PAL activity increased in root tissues of plants grown with nitrate as sole source of nitrogen and this correlated well with the increase of caffeic acid derivatives observed in roots. In the same manner the increases of chlorogenic acid, echinacoside and cichoric acid in roots of salinized plants were linked to the increase of PAL activity (Fig. 2).

Comparison of the two experiments also suggests the influence of plant age and development stage on the biochemistry of caffeic acid derivatives. In fact, higher metabolite content was found in the plants that had started to bloom (exp. N. 2), nonetheless, lower activities of both SKDH and PAL were determined in all tissues.

These findings, and those reported by Zheng et al. (2006a,b), showed that *E. angustifolia* plants, grown hydroponically under glasshouse conditions, had lower or similar amounts of active metabolites, as found in plants grown in open fields (Binns, Arnason, & Baum, 2002a; Binns, Livesey, Arnason, & Baum, 2002c). Echinacoside is a bioactive molecule, also used as a marker to distinguish *E. angustifolia* from *E. purpurea,* which has a low or negligible content of this metabolite (Binns et al., 2002c). Although the echinacoside concentration determined in our experiments and in the work conducted by Zheng et al. (2006b) are close to the minimum concentrations required by the pharmacopoeias of many countries (for instance, 0.5% dry weight in Europe; European Directorate for the Quality of Medicines and Health Care, 2002), it remained below the minimum quality standard generally established for *E. angustifolia* material for the industrial production of powdered extract that is 1.0% dry weight, according to the information provided by a few private companies.

In conclusion, the characteristics of the nutrient solutions affected the accumulation of active principles in the leaf and root tissues of *E. angustifolia* plants grown in water culture, thus suggesting that proper manipulations of the plant mineral nutrition or the induction of moderate salinity stress may provide an effective tool for producing plant material to be processed on an industrial scale.

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