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Abstract Roots encounter cinnamic acid and its hydrox- ylated derivatives that are commonly found in soils. How- ever, root systems consist of different root types with different morphological and physiological characteristics.
Very little is known about the responses and adaptation mechanisms of the root types to cinnamic acid and its hydroxylated derivatives. In this study, the morphological and physiological responses of different maize root types exposed to different concentrations of t-cinnamic, ferulic, caffeic or p-coumaric acids were investigated. The results showed that the effects of allelochemicals were dependent on concentration, chemical structure, root type and process considered. In particular, t-cinnamic acid was characterized by higher allelopathic activity when compared with its derivatives, where a hydroxyl or methyl groups were present in aromatic ring. Among root types it was possible to delineate the following tolerance hierarchy: primary > seminal > nodal > lateral of the primary = lateral of the seminal roots. Moreover, primary and seminal roots showed a different strategy to cope the chemical stress by either increasing or decreasing specific root length. Finally, an electrophysiological approach identified an involvement of proton pump activity and consequently a decrease in nitrate uptake.

Keywords: Allelochemicals, Maize, Membrane potential, Nitrate uptake, Plasma membrane, H^+-ATPase, Root morphology, Root types.

Introduction

Allelochemicals produced by plants, are secondary metabolites that can influence in positive or negative ways the growth and development of many organisms. These compounds, leached in the soil, influence root morphological and physiological processes (Einhellig 1995). Phenolic compounds are common in soils and concentrations around 90 ppm have been reported in rhizosphere soil solution (Whitehead 1964). Various phenolic compounds such as ferulic, o-hydroxyphenyl acetic, and p-coumaric acids have been isolated from decomposing rice residues in soil (Chou and Lin 1976). Moreover, cinnamic acid derivatives are commonly found in soil at concentrations between 0.01 and 0.1 mM, and affect germination and seedling growth of various species (Whitehead 1964; Macıas 1995), but the physiological role of many of them remains unknown. For example, exposure of plants to allelochemicals reduces water use (Holappa and Blum 1991), inhibits foliar expansion (Blum and Rebbeck 1989) and root elongation (Pramanik et al. 2000), and decreases nutrient uptake (Lyu and Blum 1990; Bergmark et al. 1992; Booker et al. 1992; Abenavoli et al. 2010). However, although the aerial parts are strongly influenced by allelochemicals, the root system is likely to be the first organ influenced by these compounds, interfering with its form and functions (Hartley and Whitehead 1985; Blum 1996). Many authors have observed the effects of coumarin (Svensson 1971; Aliotta et al. 1993; Abenavoli et al. 2001; Lupini et al. 2010; Lupini et al. 2014), umbelliferone (Jankay and Muller 1976; Kupidlowska et al. 1994; Abe- navoli et al. 2008), and cinnamic acids (Vaughan and Ord 1991; Yu and Matsui 1997; Abenavoli et al. 2008) on root morphology and physiology. Changes in lipid metabolism and protein synthesis of cucumber roots were observed after exposure of allelochemicals extracts from rye (Burgos et al. 2004). Caffeic acid increased the rooting of mung bean by altering enzymatic activities (Batish et al. 2008): decreasing PAL activity and H2O2 content or increasing POD activities (Bubna et al. 2011). Recently, Zanardo et al. (2009) suggested that p-coumaric acid increased the H and G lignin monomers, which solidified the cell wall and reduced soybean root growth. However, these studies have considered the root system as a single monolithic structure and this may represent a limitation because the organ actually consists of different root types with different morphological and physiological characteristics (Waisel and Eshel 2002), responding differentially to stresses (Zobel 1995; Zobel and Brown 1995).

Maize is a classic model cereal plant and it forms a complex root system composed of several different root types (Hochholdinger et al. 2004; Hochholdinger and Tuberosa 2009). Maize root system is composed by embryonic (primary and seminal) and post-embryonic (lateral and nodal) roots, which possess different functions during the life cycle of the plant (Hochholdinger et al. 2004). Embryonic roots play an essential role only in the first phase of plant development, and then these functions are replaced by post-embryonic roots (Varney and McCully 1991; Feldman 1994; Feix et al. 2002). However, among the different root types a compensatory activity is present, for example in nutrient uptake (Jeschke et al. 1997; Xu et al. 2009; Yan et al. 2011), suggesting that the longevity and importance of different root classes can alter, depending on the integrated functioning of the entire root system (Yu et al. 2014). Different responses of the root types to stress factors were also reported. Indeed, Abenavoli et al. (2004) demonstrated different responses in maize root types exposed to

coumarin. In particular, the nodal root type showed higher sensitivity than primary and seminal roots when considering root length, and this hierarchy was also observed during root tip swelling (Abenavoli et al. 2004). How the development of different maize root types is regulated by allelochemics is not well understood and, furthermore, the mechanisms underlying their responses to allelochemicals remains elusive.

Nitrogen (N) is one of the most important minerals, affecting plant growth and development and it is known to be nutrient and signal (Crawford 1995; Nacry et al. 2013). Although the allelochemical effects on nitrate uptake in whole root were studied (Booker et al. 1992; Yu and Matsui 1997; Abenavoli et al. 2010), information about the specific responses of these compounds on nitrate uptake in different root types are still largely unknown. As suggested by Santi et al. (1995), nitrate uptake could be closely correlated with plasma membrane H^+-ATPase activity (pm H^+-ATPase), which can also improve plant nutrition by enhancing the electrochemical proton gradient that drives ion transport across root cell membrane (Sze 1985; Morsomme and Boutry 2000). In turn, the apoplast acidification induced by pm H^+-ATPase activity is also correlated with net H^+ release and root growth rate (Schubert et al. 1990; Yan et al. 1992), namely acid growth theory (Rayle and Cleland 1970; Moloney et al. 1981).

To address these questions, the effects of t-cinnamic acid and its hydroxylated derivatives on root morphology were investigated, focusing on specific root length, root fineness and tissue density, as these parameters represent morphological components involved in stress responses (Ryser 2006). In addition to morphological root traits, cell plasma-membrane electrical potential differences, nitrate uptake and plasma membrane H^+-ATPase activity in each maize root type were also considered and the results are reported in this paper.

Materials and methods

Plant materials and growth condition

Maize (*Zea mays* L., cv. Cecilia, Pioneer, Italia) seeds, previously immersed in deionized water for 48 h, were germinated over aerated 0.5 mM $CaSO_4$ solution, in con- trolled conditions (continuous darkness; 24 °C and 70 % relative humidity). After 72 h, homogeneous seedlings were transferred to hydroponic culture containing 4.3 L of aerated one-fourth strength Hoagland solution composed of 0.4 mM $Ca(NO_3)_2$, 0.6 mM KNO_3, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 4.6 µM H_3BO_3, 0.9 µM $MnCl_2$, 0.08 µM $ZnSO_4$, 0.05 µM $CuSO_4$, 5 µM Na_2MoO_4, 20 µM Fe-EDTA. The pH was adjusted to 6.0 with 0.1 M KOH. The seedlings were maintained in a growth chamber at 24 ± 1°C with a 14 h photoperiod, at a photon flux density of 350 µmol m^{-2} s^{-1} at plant height and 70 % relative humidity. After 48 h, maize seedlings were transferred to the same nutrient solutions with addition 0, 25, 50, 75, 100, 125, 250, 500 or 1000 µM p-coumaric, caffeic, ferulic or t- cinnamic acids (Supplemental Fig. 1) for 48 h. Finally, to evaluate the morphological and physiological responses in different root types 0, 10, 100 and 300 µM for each allelochemical were also assayed. Nomenclature of the maize roots was

used according to Feix et al. (2002) and Hochholdinger et al. (2004), who identified primary, seminal and nodal roots. All reagents used were of the highest analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Root measurements

At the end of treatment (48 h) the plants were collected and separated into roots and shoots. Then, the roots were stained using 0.1 % (w/v) toluidine blue (Sigma-Aldrich, #89640) to improve the contrast during the scanner acquisition. Briefly, stained roots were positioned on the scanner, and an image was captured at 600 dots per inch (dpi) of resolution. The root length (cm), root volume (cm^3), root area (cm^2) were measured using WinRhizo Pro system v. 2002a software (Instruments Regent Inc., Quebec, Canada), and lateral roots number was counted manually from the image (Lupini et al. 2014). Furthermore, to determine dry weight (RDW, g) the roots were dried at 72 °C for 48 h. Finally, specific root length (SRL, root length/root dry weight, cm g^{-1}), root fineness (RF, root length/root volume, cm cm^{-3}), and root tissue density (RTD, root dry weight/root volume, g cm^{-3}) were calculated as reported by Romano et al. (2013).

Membrane potential measurements

All the electrophysiological measurements were performed on intact seedlings in root cells at 1 cm from the tip. Maize seedlings (7 days old) were previously grown in a hydroponic nutrient solution without allelochemicals for 48 h, and then were placed in a Plexiglass chamber and perfused with a basic buffer solution containing 0.5 mM $CaCl_2$, 0.2 mM KCl, 1 mM MES-NaOH (pH 6), before performing the electrode impalement of roots as described previously (Miller et al. 2001; Lupini et al. 2010). Membrane electrical potentials were measured with glass single-barreled microelectrodes back-filled with 200 mM KCl using a 70 mm long Microfil needle (WorldPrecision Instruments Inc., Stevenage, UK). The reference salt bridge was filled with 200 mM KCl in 2 % agar and was placed in the perfusion chamber close to the root. During each cell measurement the perfusion solution was changed to solution containing the same MES, K^+ and Ca+ concentrations and 100 µM p-coumaric, caffeic, ferulic or t-cinnamic acids and cell membrane potential was recorded for 10 min.

Net nitrate uptake rate in maize root types

Nitrate uptake measurements were performed on maize seedlings (7 days old) grown in a hydroponic nutrient solution and then transferred to N-free solution for 48 h (starved plant). Then, 200 µM NO_3^- was added and after 0, 4, 8, 24 and 48 h of contact the Net Nitrate Uptake Rate (NNUR) was calculated. In particular, the first 2 cm from tip of different root types were placed in the chamber (2 x 2 cm), isolated and fixed with silicone grease. Then, 1 mL of the nutrient solution containing 100 µM NO_3^- without (control) or with (treatment) 100 µM p-coumaric, caffeic, ferulic or t-cinnamic acids, was put in the chamber and samples (100 µL) were taken at 10 min intervals over a 60 min period. NO3-

concentration was measured at 210 nm with a UV–vis spectrophotometer (Perkin Elmer Lambda 35, Walthman, MA, USA). The NNUR was calculated from the linear phase of the nitrate depletion curve and expressed as µmol NO_3^- h^{-1} cm^{-1} (Sorgonà et al. 2011).

Isolation of plasma membrane vesicles

Plasma membrane (pm) vesicles were isolated at the first 2 cm from tip of different root types using a small-scale procedure from Giannini et al. (1988) modified by Santi et al. (1995). Maize seedlings were exposed to 200 µM NO_3^- without (control) or with (treatment) 100 µM p-coumaric, caffeic, ferulic or t-cinnamic acids, for 24 h. Then, maize root types (2 g) were homogenized in extraction buffer [(250 mM sucrose, 10 % (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT, 5.7 % (w/v) choline chloride, and 25 mM BTP buffered to pH 7.6 with MES, and 1 mM PMSF, and 20 mg mL^{-1} chymostatin (added before homogenization)], filtered and centrifuged twice at 12,700g for 3 and 25 min, at 4 °C. The suspension was layered over a 25/38 % discontinuous sucrose gradient [(10 mM DL-α-glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EGTA, 2 mM ATP, 1 mM PMSF, 2 mM DTT, 20 mg mL^{-1} chymostatin, 5.7 % (w/v) choline chloride, 5 mM BTP buffered at pH 7.4 with MES)] and centrifuged at 12,700g for 60 min at 4 °C. The vesicles, banding at the 25/38 % interface layers, were collected and centrifuged at 14,000g for 45 min at 4 °C. The pellet was re-suspended in a medium [(20 % glycerol (v/v), 2 mM EGTA, 2 mM EDTA, 0.5 mM ATP, 1 mM PMSF, 2 mM DTT, 20 mg mL^{-1} chymostatin, 5.7 % (w/v) choline chloride, 5 mM BTP buffered at pH 7 with MES)], and immediately frozen in liquid N2 and stored at -80 °C until use.

Plasma membrane H1-ATPase activity

ATP-hydrolyzing activity was determined by measuring the release of inorganic phosphate, as described previously (Forbush 1983). The assay medium (0.6 mL) contained 50 mM MES-BTP pH 6.5, 5 mM $MgSO_4$, 5 mM ATP, 0.6 mM Na_2MoO_4, 100 mM KNO_3, 1.5 mM NaN_3, 0.01 % (w/v) Brij58, with or without 100 µM vanadate (V_2O_5), an inhibitor of P-type H^+-ATPase (Sze 1985). Sodium azide (NaN_3, 1 mM) and potassium nitrate (KNO_3, 150 mM) were used as selective inhibitors of mitochondria and tonoplast H^+-ATPase, respectively (Santi et al. 1995). The reaction was started adding 0.5–1.5 µg of membrane protein and stopped after 30 min by a solution containing: 0.6 M HCl, 3 % (w/v) SDS, 3 % (w/v) ascorbic acid and 0.5 % (w/v) ammonium molybdate at 2 °C. The pm H^+-ATPase activity was expressed as nmol Pi $µg^{-1}$ protein h^{-1}.

Protein assay

Total soluble protein was estimated according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Plant root responses to allelochemicals were evaluated as a function of the dose by using the following nonlinear regression equation (Belz et al. 2005):

$$y = C + \frac{D - C}{1 + e^{[B \ln\left(\frac{x}{ED_{50}}\right)]}}$$

with C lower asymptote or response at indefinitely doses, D upper asymptote or mean response of the untreated control, B slope or rate of change around ED_{50}, and ED_{50} dose causing 50 % of the total response or the point of inflection. The parameters of the non-linear equations were estimated by the least squares method of non-linear regression (TableCurve 2D Version 4.0 Software, Jandel Scientific Ekrath, Germany) using the Levenburg–Marquardt algorithm.

All experiments were set up in a completely randomized design with at least five replications. All data were checked for normality (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Leven median test). The data were analyzed by one-way ANOVA, and means were separated by Tukey's honest significant difference (HSD) test (p B 0.05). Statistical analysis of the root morphology, nitrate uptake and pm H?-ATPase activity was performed by Student's unpaired t test ($p \leq 0.05$) comparing the different allelochemicals to control. Statistical analysis was employed using Systat software (Systat Software Inc., Chicago, IL, USA).

Results

Dose–response curve

In the first step, the plants were exposed to range concentration (0–1000 µM) for each allelochemic and total root length was used to estimate by non-linear equation the parameters reported in Table 1. All compounds showed a high significance p (<0.001) and correlation (>0.5) of the curve fit. Moreover, C and D parameters displayed the absence of statistical differences (Table 1), but the allelopathic potential for each compound, expressed by ED_{50} value, showed p-coumaric acid (284 µM) with a lower allelopathic effect, characterized by significant differences respect to the other compounds (128, 137 and 103 µM for caffeic, ferulic and t-cinnamic acid, respectively) (Table 1). Similar differences were also observed in the B value, where p-coumaric acid showed higher B (6.3 cm μM^{-1}), which was statistically different to t-cinnamic, ferulic and caffeic acid (2, 1.6 and 1.8 cm μM^{-1}, respectively) (Table 1).

Root types length and number

Although allelopathic stress limited the total root length of maize, to identify which root type (primary, seminal, nodal, lateral of the primary or lateral of the seminal) was influenced by different compounds, the plants were exposed to 0, 10, 100 and 300 µM for each compound, and the length of different root types was measured (Fig. 1; Supplemental Fig. 2). Primary root length did not show an inhibitory or

stimulatory effect (Fig. 1a). Seminal root length was affected only by ferulic acid, which at 100 and 300 µM significantly reduced growth by 29.7 and 40.9 % when compared to control (Fig. 1b). Nodal root lengths were reduced only by t-cinnamic acid at 10, 100 and 300 lM by 69, 64 and 56 % relative to the control, respectively (Fig. 1c). Ferulic acid at the highest concentration (300 µM) gave a significant reduction of the lateral root length of the primary roots, and this root type was also inhibited by t-cinnamic acid at all concentrations assayed (Fig. 1d). Moreover, the highest concentration (300 µM) of each allelochemical inhibited the lateral root length of the seminal roots, but t-cinnamic acid showed the same effect at 10 and 100 µM (Fig. 1e).

The number of the seminal, nodal and lateral roots of the primary did not change when exposed to allelochemicals (Fig. 2a–c). By contrast, a reduction was observed in lateral roots number of the seminal root when the plants were exposed to 100 and 300 µM ferulic (46 and 61 %, respectively), 300 µM caffeic (51 %), and 300 µM t-cinnamic (82 %) acids (Fig. 2d).

Root components

The root types were differently affected by the allelochemical treatments. Indeed, primary root dry weight was significantly reduced at all concentrations and for all allelochemicals (Fig. 3a), except 100 and 300 µM t-cinnamic acid (Fig. 3a). On the contrary, the SRL of the primary root increased by allelochemical treatments. In particular, p- coumaric, caffeic and t-cinnamic acids increased significantly this parameter at all levels, while ferulic acid only at the lowest concentration (Fig. 3b). Contrary to other allelochemicals, which did not show significant differences with respect to the control, 100 and 300 µM ferulic acid decreased the root finesses of the primary root (Fig. 3c). All allelochemicals differentially gave a reduction in the tissue density of the primary root (Fig. 3d). In particular, caffeic acid showed a reduction at 10, 100 and 300 µM of the tissue density by 49.6, 44.4, 20.5 % with respect to the control (Fig. 3d). A similar trend was also observed at 10 and 100 µM ferulic and t-cinnamic acids with 45.4 and 45.8 for the former and 52.8 and 38.2 % for the latter with respect to control (Fig. 3d). p-Coumaric acid treatment showed the same effect only at 10 µM (Fig. 3d).

Considering seminal roots, a reduction of the root weight was observed at 100 and 300 µM p-coumaric acid by 36 and 33 % with respect to the control (Fig. 3e). Moreover, the highest levels of caffeic and ferulic acids reduced the weight by 37 and 35 %, respectively, whereas no significant effects were observed with t-cinnamic acid (Fig. 3e). SRL of the seminal root was also increased at all concentrations of p-coumaric acid, while other allelochemicals did not show any significant effect (Fig. 3f). Seminal root finesses was not influenced by t-cinnamic and caffeic acids treatments, whereas only ferulic and p-coumaric acids increased this parameter at 10 µM (Fig. 3g).

This last compound decreased seminal root tissue density at 100 and 300 µM in contrast to other allelochemicals (Fig. 3h). Finally, nodal roots were unaffected by treatment with all allelochemicals (Supplemental Fig. 3).

Electrophysiology

The membrane potential steady-state (SS), before and after 10 min perfusion treatment with 100 µM allelochemicals, and the difference (D) between these values, was recorded. Before the perfusion solution, all root types showed similar membrane potentials at SS (Table 2), whereas the time- course measurements of Vm recorded a strong and rapid depolarization (less negative potential) occurring a few minutes after contact with the allelochemicals (Table 2). In primary roots, the depolarization with t-cinnamic acid (19 mV) was significantly higher than p-coumaric (15 mV), ferulic (13 mV) and caffeic (9 mV) acids (Table 2). Although a similar trend was also observed in seminal and nodal roots, the value of the depolarization was different (Table 2). Indeed, 100 µM t-cinnamic, p-coumaric, ferulic or caffeic acids in seminal roots showed the following depolarization 44, 24, 28 and 11 mV (Table 2), while 22, 16, 14 and 11 mV was observed in nodal roots, respectively (Table 2).

Net nitrate uptake rate measurements

The time-course of the NNUR of the three root types (primary, seminal, and nodal) exposed to 200 µM nitrate for 48 h, was measured to give the maximum uptake (full nitrate uptake induction) on which to determine then the effects of the different allelochemicals. In the different root types exposed to nitrate the net nitrate uptake rate progressively increased reaching a peak of maximum activity after 24 h. Thereafter, a subsequent decline of NNUR in the three root types was observed (data not shown). Using 100 lM for each allelochemical and 24 h as induction time, the allelopathic effects on NNUR were evaluated and reported in Fig. 4. In the primary root, p- coumaric acid reduced NNUR by 62.7 % with respect to the control (Fig. 4), whereas the other allelochemicals did not show any significant difference (Fig. 4). A different behavior was observed in seminal roots, where p-coumaric, ferulic and caffeic acids showed a NNUR decrease of 39.1, 40, 45.5 % with respect to the control (Fig. 4). Finally, NNUR in nodal roots was decreased only with t-cinnamic acid (44 %), whereas there was an increase (37 %) shown when the roots were exposed to p-coumaric acid (Fig. 4).

Plasma membrane H1-ATPase activity

After 24 h of exposure to 200 µM nitrate without (control) and with 100 µM t-cinnamic, ferulic, caffeic or p-coumaric acids, the pm H^+-ATPase in different maize root types was determined and the results are reported in Fig. 5. In the primary root the enzyme activity was significantly inhibited by t-cinnamic acid (43 % respect to control), whereas other allelochemicals did not show significant difference (Fig. 5). The exposure of the seminal root to t-cinnamic, caffeic and ferulic acids determined a reduction of the pm H^+-ATPase activity by 54, 37 and 36 % respect to control, respectively (Fig. 5). Finally, all allelochemicals assayed reduced the enzyme activity in nodal root, respect to control (Fig. 5).

Discussion

The present study demonstrates that cinnamic acid and its derivatives significantly affected morphological and physiological processes in maize roots. Furthermore, this study shows that the specific response of maize root to allelochemicals depends on the developmental type of root and thereby indicating the difficulties of averaging whole roots and treating them as a single structure. Different types of maize root possess specific morphological, physiological and molecular characteristics (McCully 1999; Waisel and Eshel 2002; Feix et al. 2002; Hochholdinger et al. 2004; Kong et al. 2014), determining different responses to allelochemicals. The effects of allelochemicals were pre- viously evaluated in either embryonic or post-embryonic roots. The results based on averaging whole root systems have identified that the effects of allelochemicals were dependent on concentration and chemical structure of the compound. Indeed, t-cinnamic acid showed lowest ED50 value (Table 1), compared to p-coumaric, caffeic, ferulic acids, which are characterized by the presence of substi- tutions in the aromatic ring. These results are in agreement with previous works, which reported that the presence of substitutions in the aromatic ring changed the allelopathic activity (Yu and Matsui 1997; Abenavoli et al. 2010; Jitareanu et al. 2013). Furthermore, Wong et al. (2005) demonstrated that also the position and/or the isomerization of the substitution group defined the chemicals allelopathic activity. For example, cis-cinnamic acid was more active than t-cinnamic acid in root growth inhibition of *Arabidopsis thaliana*. In addition, considering the different chemical proprieties of the allelochemicals used in the present work, it is possible to delineate a correlation with the morphological effects. Indeed, as suggested by Yu and Matsui (1997), the lipophilicity could represent a marker in the allelochemical responses. Our results corroborate this hypothesis, as t-cinnamic acid is characterized by the higher lipophilicity and toxicity respect to other allelochemicals used.

On the other hand, the analysis of different root types has permitted the identification of differing tolerances among the root types to the compounds tested. In particular, lateral root length in primary and seminal roots was characterized by a higher sensitivity, reducing root length (Fig. 1) and number (Fig. 2), when compared with other root types. These different responses may be interpreted by their diverse anatomy (Luxova` and Kozinka 1970; Wang et al. 1995; McCully 1999; Hochholdinger et al. 2004), possibly facilitating differential penetration and translocation of the allelochemicals across the root membrane. In fact, lateral roots differ from others as they are more sensitive to drying by transpiration rate changes (Wang et al. 1991) and have a dominant role in water uptake (Wang et al. 1995; Hochholdinger et al. 2004). Moreover, the number and lateral root length are strongly dependent on the plant hormone auxin, which plays a central role in organ development and elongation, in shoot/root branching and plastic growth responses (Zazımalova´ et al. 2010), in lateral root initiation (Casimiro et al. 2003; De Smet et al. 2006), primordia development (Benkova´ et al. 2003) and emergence (Laskowski et al. 2006). Furthermore, t-cinnamic acid is a potent auxin inhibitor (Jitareanu et al. 2013) as are other cinnamic acid derivatives, and these could interfere with auxin transport and/or distribution, reducing the lateral root number.

As root components are traits involved in stress responses, the effects of the allelochemicals on root types were also evaluated for different components of the whole root morphology, as specific root length (SRL), fineness (F), and tissue density (TD). The results showed that primary and seminal roots

displayed different strategies to cope allelopathic stress: SRL was increased in primary root, whereas seminal root did not show significant differences. As reported by Romano et al. (2013), SRL changing may be due to variation in F or TD (Romano et al. 2013) and our data showed a decrease in primary root TD. Thus this study shows evidence for the complexity of effects of phenols on root morphological parameters, which can be inhibitory, stimulator or ineffective. Maffei et al. (2007) suggested that the plasma membrane electrical potential represents the earliest cellular sensing element to abiotic stresses. Measurements of the electrical potential difference in each root type exposed to allelochemicals were employed (Table 2). The size of the cell membrane electrical potential difference before the treatment indicated the energy status of the plasma mem- brane and it is important for driving transport and growth: a larger potential (more negative) suggesting more energy is available for plasma membrane transport. The different root types did not show any differences, before the allelochemical treatments, but a different depolarization was observed among root types after few minutes of exposure (Table 2). The present results are in agreement with those of Glass and Dunlap (1974), which showed that cinnamic acids caused a cell membrane depolarization, supporting the hypothesis that these compounds influenced membrane permeability. Moreover, as reported by Brault et al. (2004), the membrane depolarization may indicate an inhibition of the plasma membrane H^+-ATPase. Our results confirm this hypothesis, as plasma membrane H^+-ATPase activity was reduced when the plants were exposed to allelochemicals, and these results were also confirmed in previous studies, which demonstrated that cinnamic acid derivatives reduced enzyme activity (Abenavoli et al. 2010).

Moreover, nitrate uptake by roots requires energy to overcome the negative electrical potential across plasma membrane of root cells (Miller and Smith 1996; Miller et al. 2001), and the co-transport with protons is provided with energy by the activity of the plasma membrane H^+- ATPase. For this reason, we speculated that reduction observed of the proton pump activity might also provide a mechanism for the inhibitory effects on nitrate uptake by allelochemicals.

Finally, as suggested by many authors, a strong correlation was delineated between root growth and plasma membrane H^+-ATPase (Yan et al. 1992). From our results, a mechanism action of t-cinnamic acid and its derivatives in maize root types, based on the classic acid growth theory, could be proposed. Allelochemicals interacts with plasma membrane causing an immediate transient depolarization and then subsequently inhibited the pm H^+-ATPase activity resulting in a decreased H^+ efflux and increased pH of the apoplast, thereby reducing root growth rate.

In conclusion, the results of this investigation confirm the hypothesis that cinnamic acids exert a significant allelopathic action on maize root types by interfering with several morphological and physiological processes. In particular, the results suggest that the effects of allelochemicals have many targets, depending on the chemical structure, plant species, root type and physiological processes considered. These results reveal that distinct patterns in the different types of maize roots occur in responses to allelochemicals and these may play important roles in the development and growth responses to environmental cues.

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Table 1 Parameters estimated by non linear equation in maize roots exposed to allelochemicals for 48 h

Compound	ED_{50} (μM)	B (cm μM^{-1})	C (cm)	D (cm)	R^2	*p*
p-Coumaric acid	284^a (34)	6.3^a (1.7)	76^a (5.8)	247^a (3)	0.782	0.00001
Ferulic acid	137^b (29)	1.6^b (0.1)	66^a (11)	262^a (0.2)	0.528	0.00001
Caffeic acid	128^b (22)	1.8^b (0.5)	65^a (6.8)	279^a (14)	0.705	0.00001
t-Cinnamic acid	103^b (29)	2^b (0.4)	51^a (12)	220^a (14)	0.577	0.00003

ED_{50} dose causing 50 % of the total response or the point of inflection, B slope or rate of change around ED_{50}, C lower asymptote or response at indefinitely large doses, D upper asymptote or mean response of the untreated control, R^2 coefficient of determination, p significant level. Different letters along the columns indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$ ($n = 10$)

Table 2 Electrophysiological responses of maize root types to 100 µM allelochemicals

Compound	Root type								
	Primary			Seminal			Nodal		
	SS	10 min	Δ	SS	10 min	Δ	SS	10 min	Δ
p-Coumaric acid	−131a	−116	15b	−122a	−98	24b	−111a	−95	16b
Ferulic acid	−128a	−115	13b	−129a	−101	28b	−104a	−90	14b
Caffeic acid	−123a	−114	9c	−133a	−122	11c	−109a	−98	11c
t-Cinnamic acid	−130a	−111	19a	−120a	−76	44a	−104a	−82	22a

SS steady state, 10 min after 10 min from allelochemics perfusion, D differences between 10 min and SS. Different letters along the columns indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$ ($n = 8$)

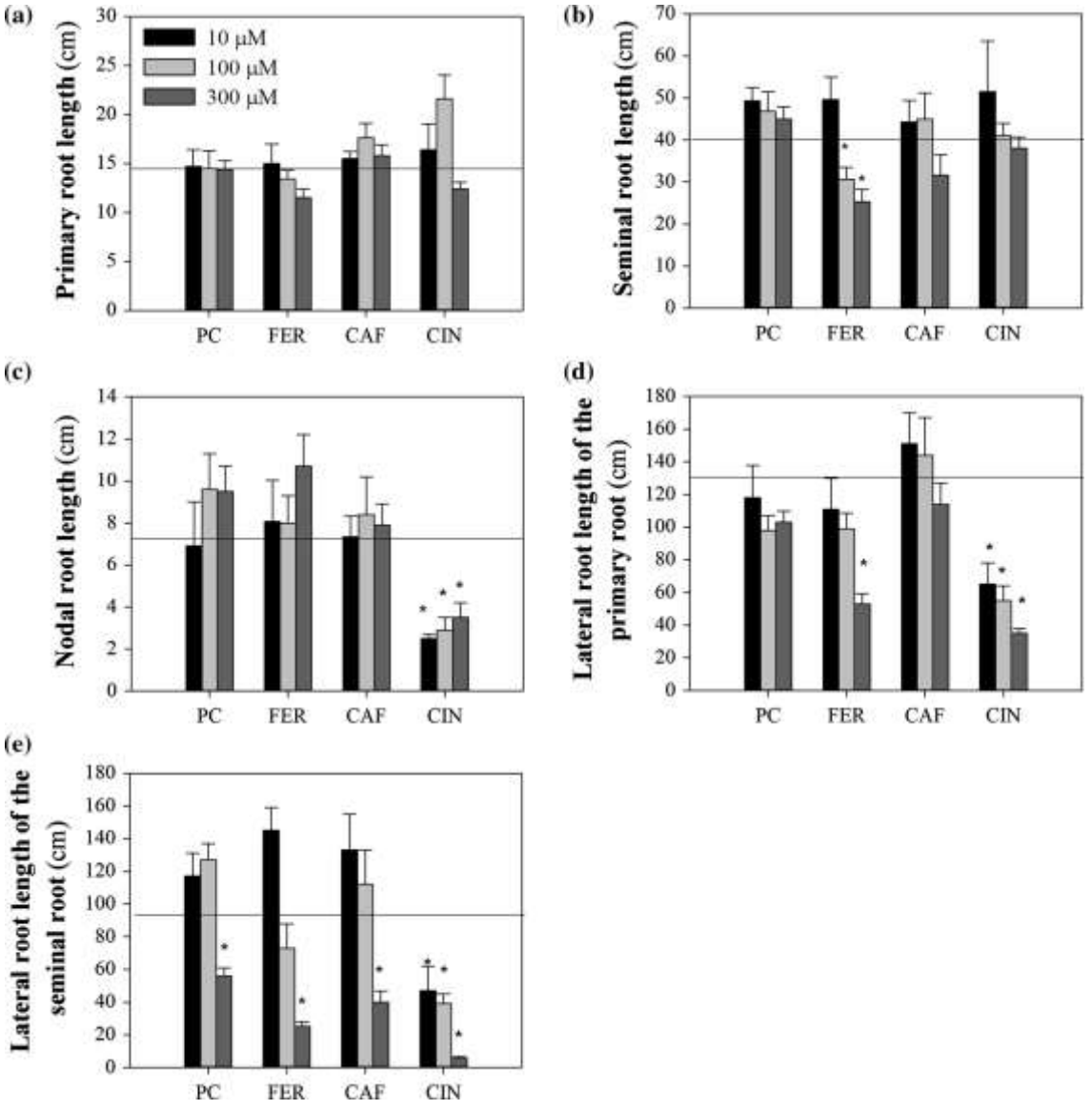


Fig. 1 Root length of maize root types (a primary, b seminals, c nodal, d lateral of the primary, e lateral of the seminals) exposed to the allelochemicals, p-coumaric (PC); ferulic (FER), caffeic (CAF), t-cinnamic (CIN), acids for 48 h. The values are showed as mean ± SE (n = 7). *Significant different at $p < 0.05$, respect to control (line), according to Student's unpaired t test

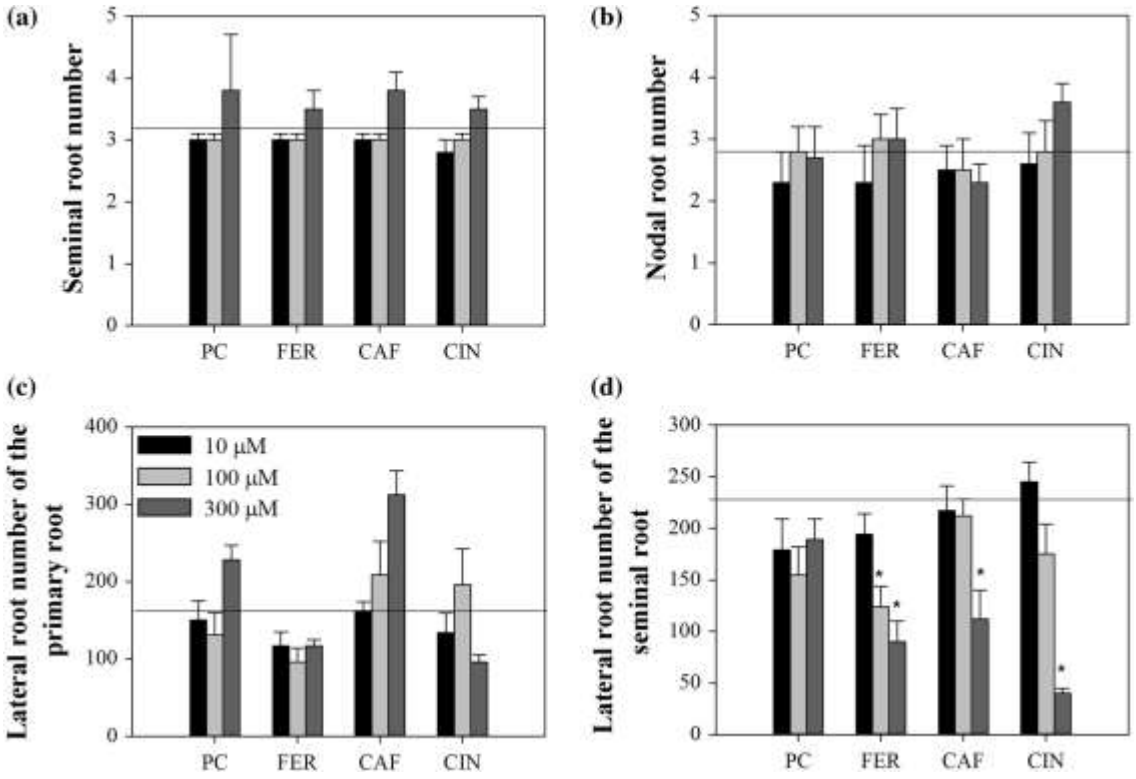


Fig. 2 Root number of maize root types (a seminals, b nodals, c laterals of the primary, d laterals of the seminals) exposed to the allelochemicals, p-coumaric (PC); ferulic (FER), caffeic (CAF), t-cinnamic (CIN), acids for 48 h. The values are showed as mean ± SE (n = 7). *Significant different at $p < 0.05$, respect to control (line), according to Student's unpaired t test

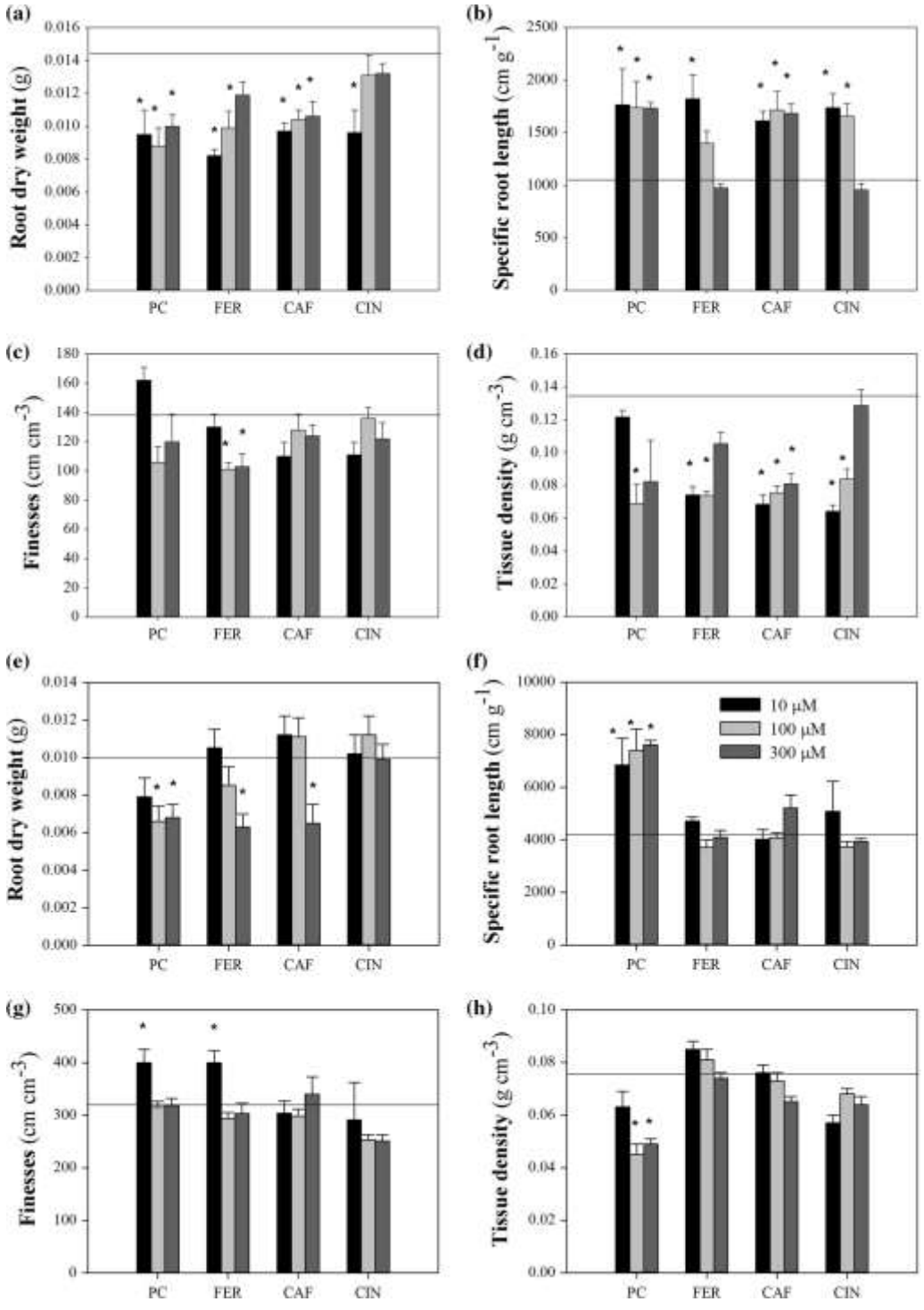


Fig. 3 Primary (a–d) and seminal (e–h) roots exposed to p-coumaric (PC); ferulic (FER), caffeic (CAF), t-cinnamic (CIN), acids for 48 h. The values are showed as mean ± SE (n = 7). *Significant different at $p < 0.05$, respect to control (line), according to Student's unpaired t test

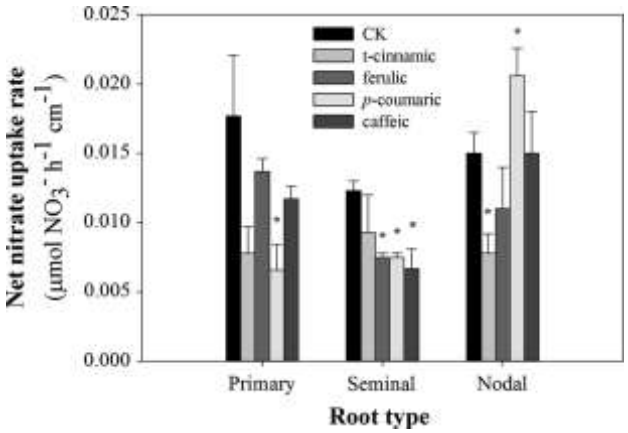


Fig. 4 Net nitrate uptake rate in different root types (primary, seminal and nodal) of maize, 7 days old, exposed to 200 μM NO - and 100 μM of different allelochemicals for 24 h. The values are showed as mean ± SE (n = 5). *Significant different at $p < 0.05$ respect to control (CK), according to Student's unpaired t test

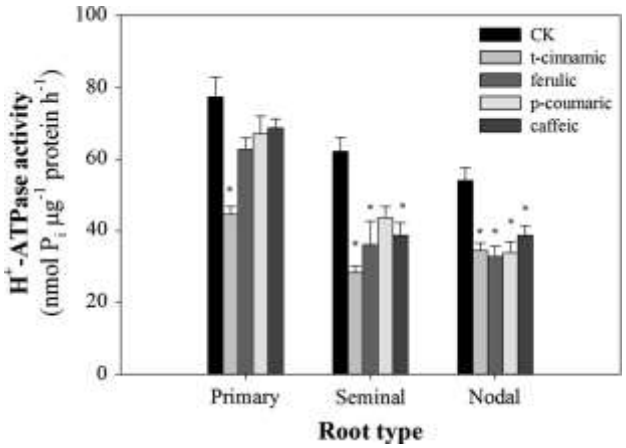
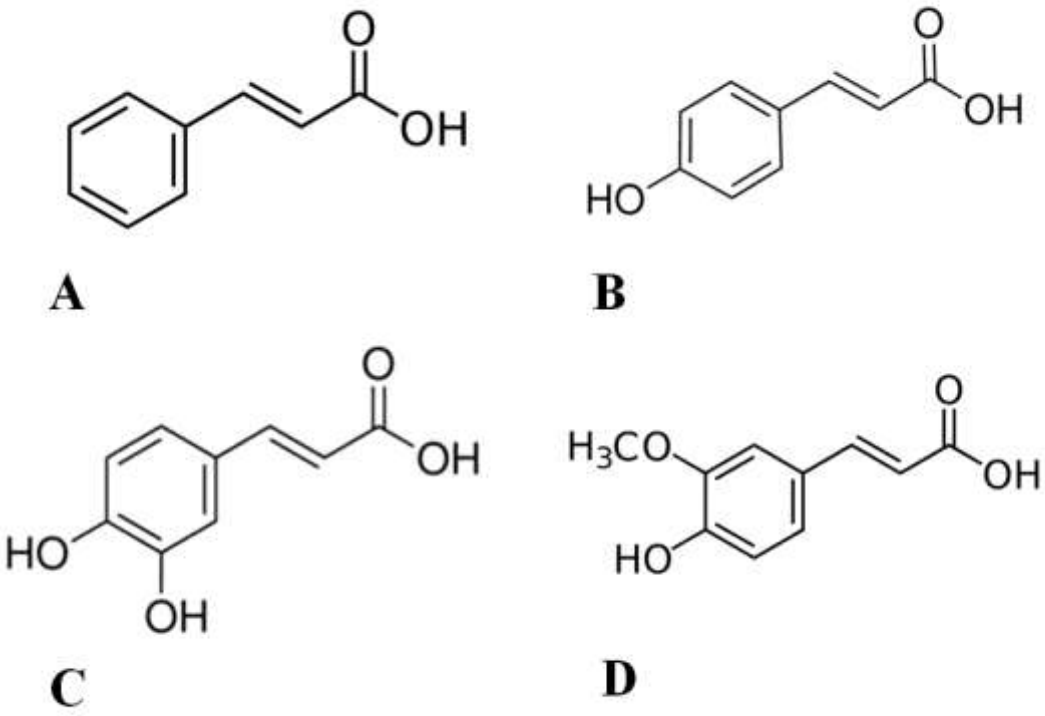
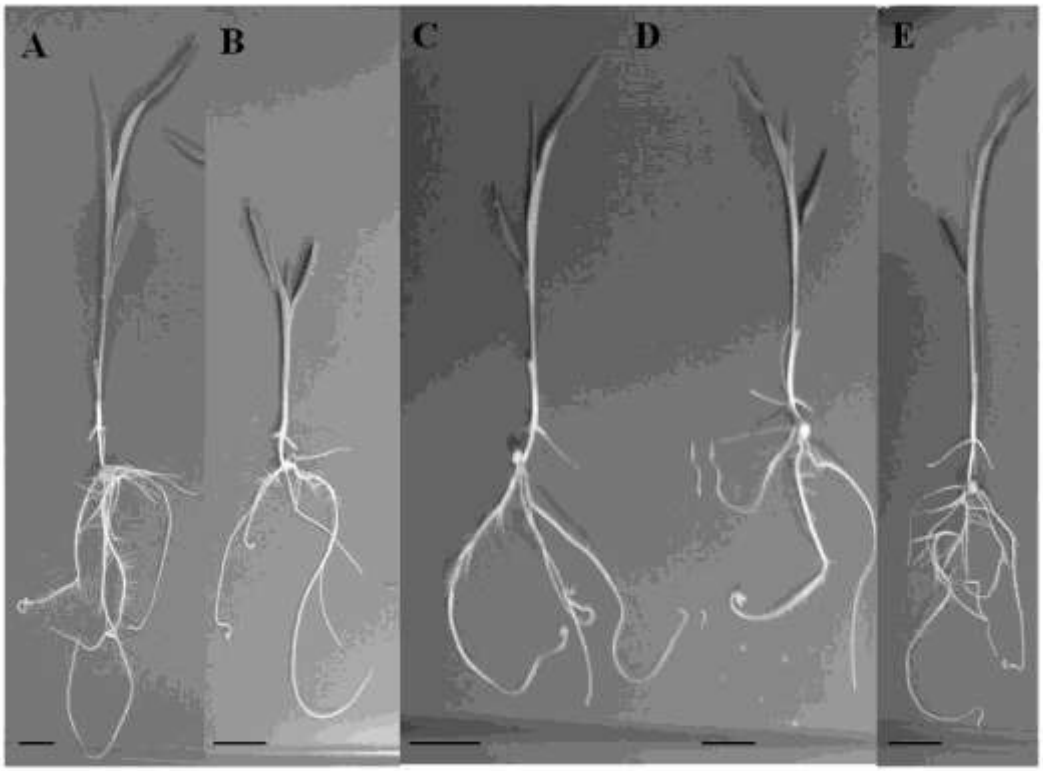


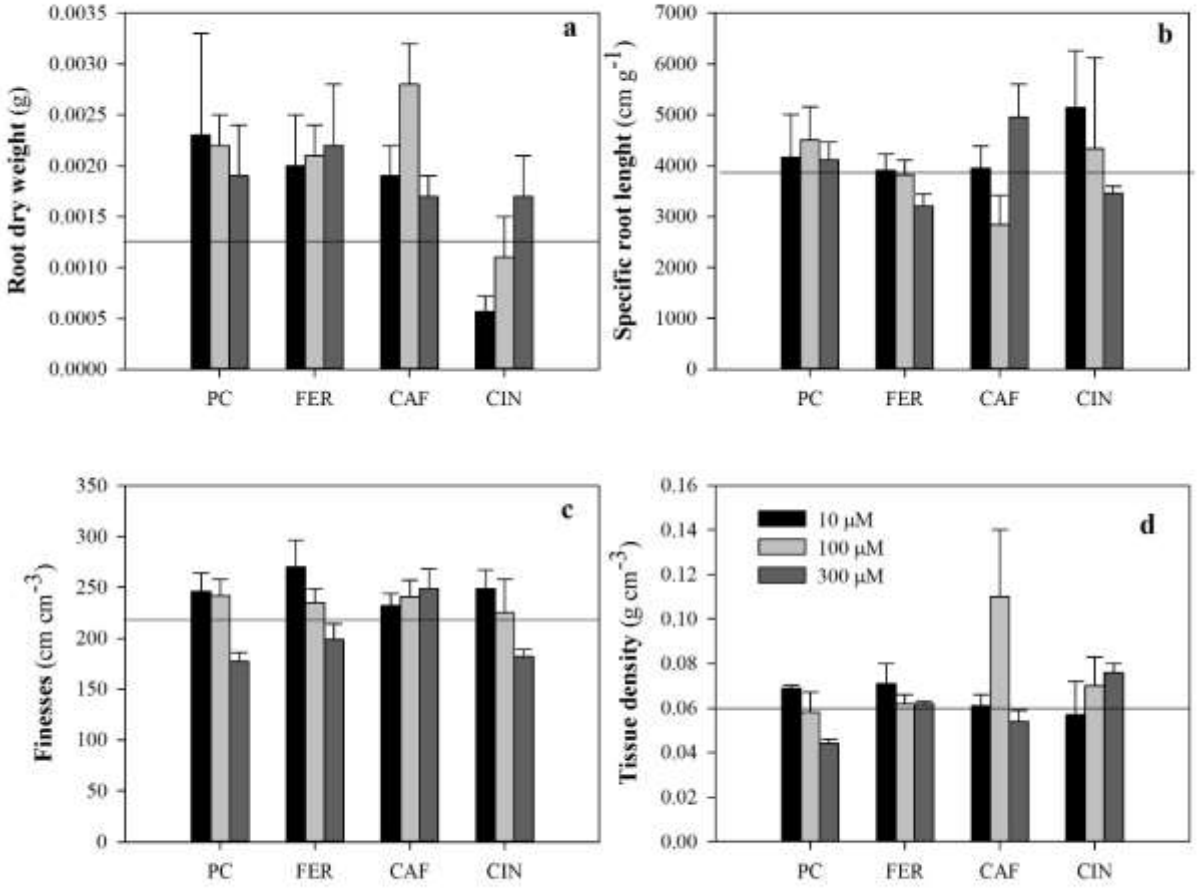
Fig. 5 H^+-ATPase activity (nmol Pi μg^{-1} protein h^{-1}) of plasma membrane vesicle isolated from different maize root types exposed to 100 µM different allelochemicals for 24 h. The values are showed as mean ± SE (n = 5). *Significant different at p \ 0.05 respect to control (CK), according to Student's unpaired t test



Supplemental Fig. 1 Compounds used in this study. A, t-cinnamic acid; B, p-coumaric acid; C, caffeic acid; D, ferulic acid



Supplemental Fig. 2 Representative pictures of maize seedlings exposed to 300 μM allelochemicals for 24 h (A, control; B, t-cinnamic acid; C, caffeic acid; D, ferulic acid; E, p-coumaric acid)



Supplemental Fig. 3 Nodal roots exposed to the allelochemicals, p-coumaric (PC); ferulic (FER), caffeic (CAF), t-cinnamic (CIN), acids for 48 h. The values are showed as mean ± SE (n = 7). * = significant different at $p < 0.05$ respect to control (line), according to Student's unpaired t test