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# Root plasticity of *Nicotiana tabacum* in response to phosphorus starvation

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## Abstract

Tobacco plants under low phosphate exhibited increased total and tap root length, as a result of higher apex activity, but decreased root branching in comparison to the plants grown with high-Pi. The possible mechanisms and significance of these alterations, which differed from those typical of stress-induced morphogenetic responses, are discussed.



**Keywords:** root system architecture, root apices, phosphate, cadmium, stress, *Arabidopsis,* auxin, arbuscular mycorrhizae.


## Introduction

Plant development is highly variable and strongly influenced by environmental signals. In particular the root system is highly plastic, and this plasticity allows the plant to optimize the acquisition of the resources from the soil (Malamy 2005; Barlow 2010) or to cope with stressful conditions (Potters et al. 2007; 2009). Root architecture allows plants to compete effectively for resources and survive to nutrient starvation, and is thus central to reach optimal plant growth and yield (Malamy 2005; Den Herder et al. 2010). Among soil nutrients which significantly impact plant growth and health, phosphorus is one of the most important. However, it is largely unavailable for plant uptake (Vance et al. 2003). Plants respond to phosphate (Pi) starvation by altering their biochemistry, physiology and morphology in order to conserve Pi, to enhance Pi acquisition and to extend the exploration of the soil (Vance et al. 2003; Hammond and White 2008). A common response of plants grown in Pi limiting conditions is an increased root-to-shoot biomass ratio in relation to the non-starved counterparts (Ramaekers et al. 2010). In addition, plants may modify root system architecture. Some of them, such as *Arabidopsis thaliana* (López-Bucio et al. 2002; Sánchez-Calderón et al. 2005) and some *Brassica* cultivars (Akthar et al. 2009), respond to Pi-shortage by producing a shorter and more branched primary root. In other species, instead, primary (or adventitious) root length is increased or is unchanged by low Pi conditions; furthermore, unlike *Arabidopsis*, some plants show a lower degree of branching than those grown with higher Pi levels (reviewed by Fusconi 2014). However, despite the abundant literature dealing with Pi effect upon root morphogenesis, there is still a considerable incoherence often resulting from differences in experimental designs and/or morphological parameters considered.

Chronic stresses also, including metal toxicity, have shown to affect root morphogenesis even when they enable plants to survive and grow (Potters et al. 2007; 2009). These stresses induce a specific "Stress-Induced Morphogenetic Response" (SIMR, Potters et al. 2007) of the whole plant, which consists in an active and orchestrated redistribution of growth, rather than in a simple cessation of it, as a consequence of toxicity. In the roots, SIMRs lead to primary roots shorter than those of the non-stressed controls, following a block of meristem activity and inhibition of cell elongation, and to increased branching (Potters et al. 2007). In order to extend our knowledge on the influence of Pi nutrition on root system architecture, we studied the effects of Pi availability on tobacco (*Nicotiana tabacum*) plants using a culture method similar to those commonly used for *Arabidopsis*. We chose tobacco because it is a model plant of agronomic interest and easily cultivable in Petri plates for a relatively long period, whose root morphogenesis in responses to Pi availability is still little known. In addition, we analyzed the effects on root morphogenesis of cadmium (Cd), a heavy metal widespread in the environment, non-essential but readily taken up by the roots, whose toxicity on plants (Sanità di Toppi and Gabbrielli 1999; Hattab et al. 2009; Garg and Bhandari 2013) and effects on root morphogenesis (Hu et al. 2013) are well documented. Results obtained in tobacco are discussed by comparison with *A. thaliana*, in consideration of the huge amount of data available for this plant on the regulation of root morphogenesis under low Pi condition and other stresses, including Cd toxicity.

## Materials and methods

### *Plant material and growth conditions*

Seeds of *Nicotiana tabacum* L. var. Petit Avana SR-1 obtained from A.R.S. (Agricultural Research Service, USA) were surface-sterilized for 5 min. in 1% sodium hypochlorite solution and sown in square Petri plates (120×120×17mm, four seeds per plate) containing half-strength Murashige and Skoog (½ MS) media deprived of Pi (P- treatment) or with 1mM Pi as KH2PO4/K2HPO4 (P+ treatment). The growth media were solidified with 1% agar (A-1296, Sigma-Aldrich, containing about 20μM P). The strength of the MS medium and Pi concentrations were chosen on the basis of preliminary experiments. The plates were placed almost vertically in a growth chamber, without shading the roots, under controlled conditions (16/8h light/dark photoperiod, 25/22±1°C thermoperiod, 150μmol·m-2sec-1), to allow root growth along the surface of the agar.

***Ponderal and morphometric analyses***

After 28 days from germination, the dry weights of the shoots and roots of 15 plantlets were determined, and the root-to-shoot ratio was calculated. Images of the root system of 12 seedlings per treatment were recorded weekly up to 28 days from germination, by placing the Petri dishes, with a metric reference, directly onto a scanner Epson Perfection 1240U (resolution: 400dpi, 800dpi for root apex magnifications). The leaves were detached and scanned. The numbers of 1st order lateral roots and leaves were counted. The length of the roots of each order and the leaf area were measured using the image analysis software Lucia G (LIM, Praha, CZ); the degree of root branching (i.e. the number of lateral roots per length of the tap root) was calculated.

***Cortical cell length , root apex structure and activity***

i) The length of 100 mature cortical cells per treatment of the tap roots was determined, with an ocular micrometer scale, on freehand longitudinal sections taken immediately above the differentiation zone after 28 days of culture. ii) The viability of the root tips of five plants per treatment was determined by using nitro blue tetrazolium salts (NBT, Sigma-Aldrich), according to Connolly and Berlyn (1996). The percentage of active apices of the tap roots and of the most developed laterals was calculated 7, 14, 21 and 28 days after germination. The length of NBT stained apices of the tap roots was measured with an ocular micrometer scale. iii) The structure of the apical meristems and the localization of proliferating cells of the tap root apices were assessed on 21 and 28 days old plants. Root apex structure was analyzed on median longitudinal sections (MLSs), stained with toluidine blue, of epon-araldite embedded samples processed according to the usual cytological techniques. Proliferating cells were localized through indirect immunofluorescence of 5-bromo-2'-deoxyuridine (BrdU, Sigma- Aldrich) on MLSs of root apices embedded in London Resin White (Sigma-Aldrich), according to the procedure described by Fusconi et al. (2007). BrdU was localized with an anti-mouse IgG Cy3-coniugated (Sigma-Aldrich) and sections were counterstained with 4’,6-diamidino-2-phenilindole (DAPI, Sigma-Aldrich). All samples were observed with a Nikon Eclipse E400 (Nikon Corporation, Tokio, Japan) light microscope and images were acquired with a digital camera Nikon Ds-5M. Immunostained sections were observed with the same microscope equipped with epifluorescence; the UV2A and G2A filters were used for DAPI and Cy3, respectively.

### *Treatments with cadmium*

Tobacco is a known Cd-tolerant plant (Martins et al. 2011) and preliminary experiments showed that, using the above described ½ MS medium, only very high Cd concentrations modified root system morphogenesis. Therefore, a poor MS nutrient medium was prepared in accord to Quaghebeur and Rengel (2003). Seeds of *N. tabacum*, sterilized as described above, were sown on Cd-free medium. After germination they were transferred to Petri dishes containing 0; 0.2; 2; 20; 50µM of cadmium chloride (CdCl2, Sigma-Aldrich) for further 21 days. Morphometric analysis (leaf area, tap root and total root length and degree of branching) were performed as previously described.

### *Statistical analysis*

The data were expressed as means ± SE and compared through the Kruskal-Wallis oneway analysis of variance using the Systat 11 software for Windows; the differences were considered as statistically significant for $P<0.05$.

## Results and discussion

Tobacco plants grown on the P+ medium had larger and more numerous leaves and a higher total leaf area than those grown in P- conditions (Figs 1a-c); the leaf parameters increased regularly up to end of the experiment in both treatments (Fig. 1c and table I). The root-to-shoot dry weight ratio increased significantly in the P- plants in comparison to P+ plants after 4 weeks of culture (table I) as frequently occurs when plants grow under Pi starvation conditions (Ramaekers et al. 2010). These results thus pointed to a "normal" growth response of tobacco plants to Pi availability, despite the *in vitro* growth conditions used for this work. The root system of *N. tabacum* consisted of a tap root that branched mostly in its upper part (Figs 1a, b). The length of the whole root system was higher, by about 35%, at the end of the experiment in the P- plants (Fig. 1d). The tap root length of the P- plants increased linearly; on the contrary lengthening declined after 14 days of culture in the P+ treatment and was very low between 21 and 28 days of culture (Fig. 1e). The number of lateral roots was significantly higher in the P+ plant at the end of the experiment (Table I) and the degree of branching of the tap root was significantly higher in P+ plants from 14 days onwards (Fig. 1f). Root system of tobacco, therefore, responded to Pi availability differently from that of *Arabidopsis***,** where shorter and more branched primary roots were found in response to low Pi (López-Bucio et al. 2002; Sánchez-Calderón et al. 2005). Since root growth depends on cell elongation and root apex activity, we determined the

length of the cortical cells, as well as root apex morphology and activity in Pi-starved and Pisufficient tobacco plants (table II; Fig. 2). These analyses showed that, at the end of the experiment, the fully elongated cortical cells were about 9% shorter in the P+ plants than in the P- plants (132.62±2.48 and 145.77±2.59 μm, respectively). Root apices of the tap root and of the most developed laterals of P- plants generally had progressively longer root hairs, starting from the root apex base shootwards (Fig. 2a) and stained intensely with NBT (Fig. 2b, Table II) showing to be metabolically active. Instead, in the P+ treatment, most apices showed fully elongated root hairs close to the root tip (Fig. 2c) and were metabolically inactive, being slightly or not stained by NBT (Fig. 2d, Table II). The mean length of the P+ apices of the tap roots increased up to 14 days from germination thereafter attaining a mean length sensibly shorter than that of P- plants which instead lengthened up to the 3rd week of culture (Table II). These findings led us to conclude that root shortening in the P+ plants was mostly dependent on the reduced meristem activity, as the slight decrease of cortical cell length may have influenced root length only to a small extent. Observations on MLSs confirmed that in P- plants almost all the apices were active as they showed a normal organization of the meristem and a well developed and pointed root cap (Fig. 2e, e'); labeled nuclei were evenly distributed in the cortex and central cylinder meristems, apices had a defined quiescent centre and labeled initials of the root cap (Figs 2f, f'). On the contrary, in the P+ plants most apices were inactive. They frequently had vacuolated initial cortex cells and a reduced root cap (Fig. 2g). Moreover, proliferative activity decreased in most apices from 21 days onwards and about 25% of the apices almost completely lacked BrdU labeled nuclei, and were hence differentiated, 28 days from germination (Figs 2h, h'). Interestingly, the cytological alterations occurring in the root apices of tobacco under high Pi were quite similar to those leading to determinate primary root growth under low Pi in *Arabidopsis* (Sánchez-Calderón et al. 2005). These different responses are difficult to explain. In *Arabidopsis* root morphogenesis has been deeply analyzed in the last years and an important role of auxin transport and sensitivity has been recognized in the responses to Pi starvation (Pérez-Torres et al. 2008; Chiou and Lin 2011; Miura et al. 2011). On the contrary there is insufficient literature, if any, on the role of auxin metabolism in tobacco, in relation to Pi availability. Differences in IAA levels (Torelli et al. 2000) and in the expression of genes involved in auxin biosynthesis and signaling (Li et al. 2012) were found between Pi-starved and Pi-sufficient plants of *Allium porrum* and *Zea mays*, respectively. Because both plants, like tobacco, exhibit increased root length and decreased branching in low Pi conditions (Trotta et al. 1991; Li et al. 2012), these results point to a general

role of auxin in the morphogenetic responses of the root system to low Pi conditions. Auxin has also been shown to be strictly involved in the SIMR induced by toxic elements (Potters et al. 2007; 2009; Hu et al. 2013). In tobacco, as generally occurs, Cd reduced plant growth and modified root system morphogenesis (Fig. 3a). Leaf area was significantly decreased by 20 μM Cd, and was drastically reduced by 50 μM Cd (Fig 3b). The total root growth and the lengthening of the tap roots were significantly inhibited by 2 μM Cd, and this inhibition was concentration-dependent, the tap root lengthening being almost completely blocked by the highest concentrations of Cd (Figs 3c, d). Although Cd reduced tap root length, the formation of new lateral roots was less affected (10.57±0.48 and 10.59±0.54 in controls and 50 μM treated plants, respectively, after 21 days of treatment), thus resulting in higher tap root branching (Fig 3e). Treatment of tobacco plants with relatively high concentrations of Cd thus led to a typical SIMR, which has been interpreted as a way to limit stress exposure. In *Arabidopsis*, Cd treatment decreases the level of auxin near the primary root tip, causing a block of meristem cell division and root growth, whilst auxin increases in the middle and upper zone of the root were lateral roots form (Potters et al. 2007). A similar mechanism could be active in the Cd-treated tobacco plants analyzed in the present work. Indeed, redistribution of auxin seems to generally occur after Cd treatment, as recently shown also in rice plants treated with Cd (Zhao et al. 2012). In conclusion, the above data indicate that while the SIMR phenotype is similar in tobacco and *Arabidopsis*, Pi-deficiency gives rise to a root morphogenesis which changes from species to species. This may be related to the different strategies that plants have evolved to acquire Pi. In *Arabidopsis* the reprogramming of root development under Pi deprivation leads to a shallow and superficial root system to optimize the absorption of Pi, which is usually more abundant near the soil surface (Vance et al. 2003; Hammond and White 2008). Tobacco under Pi-deficiency instead lengthens its roots to enhance the possibility to encounter zones of high Pi availability (Ramaekers et al. 2010) and reduces branching probably to limit the cost of soil exploration. These contrasting responses to Pi availability may also rely on the different dependency of plant species on arbuscular mycorrhizal (AM) symbiosis. This is a wide-spread symbiosis between the roots of most land plants and Glomeromycota fungi where the fungus assists the host plant in acquiring nutrients, mainly Pi, from the soil (Smith and Read 2008; Perotto et al. 2013). Tobacco, differently from *Arabidopsis*, is an AM-host plant. It is thus tempting to speculate that the root architecture typical of tobacco (and other mycorrhizal host plants) under low Pi conditions may positively influence the colonization process. The finding that the production of strigolactones, which favour colonization by inducing AM hyphal branching (Akiyama et al. 2005), increases in Pi-starved plants (see Yoneyama et al. 2012) and that strigolactones modify root morphogenesis (Ruyter-Spira et al. 2011;

Arite et al. 2012) through changes in the regulation of auxin efflux (Koltai et al. 2010) supports the notion of a tight correlation between root morphogenesis under low Pi and the colonization process.

## Acknowledgements

This work was funded by the Italian MURST under grant 2007PKFAAT.

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Table I. Ponderal and morphometric parameters of *Nicotiana tabacum* plants grown in low and high-phosphate conditions, 28 days after germination.

| | P+ | P- | P<0.05 |
|---|---|---|---|
| Shoot dry weight (mg) | 11.08±0.75 | 6.98±0.25 | * |
| Root dry weight (mg) | 3.78±0.38 | 4.56±0.58 | - |
| Root-to-shoot ratio (mg/mg) | 0.34±0.02 | 0.65±0.07 | * |
| Leaf number | 6.50±0.31 | 4.07±0.27 | * |
| Lateral root number | 13.36±0.79 | 10.50±0.64 | * |

Table II. Percentage of active apices and root apex length in *Nicotiana tabacum* plants grown in low and high-phosphate conditions. * indicates significant differences between treatments.

| | P+ | P- | P<0.05 |
|---|---|---|---|
| Shoot dry weight (mg) | 11.08±0.75 | 6.98±0.25 | * |
| Root dry weight (mg) | 3.78±0.38 | 4.56±0.58 | - |
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| Leaf number | 6.50±0.31 | 4.07±0.27 | * |
| Lateral root number | 13.36±0.79 | 10.50±0.64 | * |

**Figures**

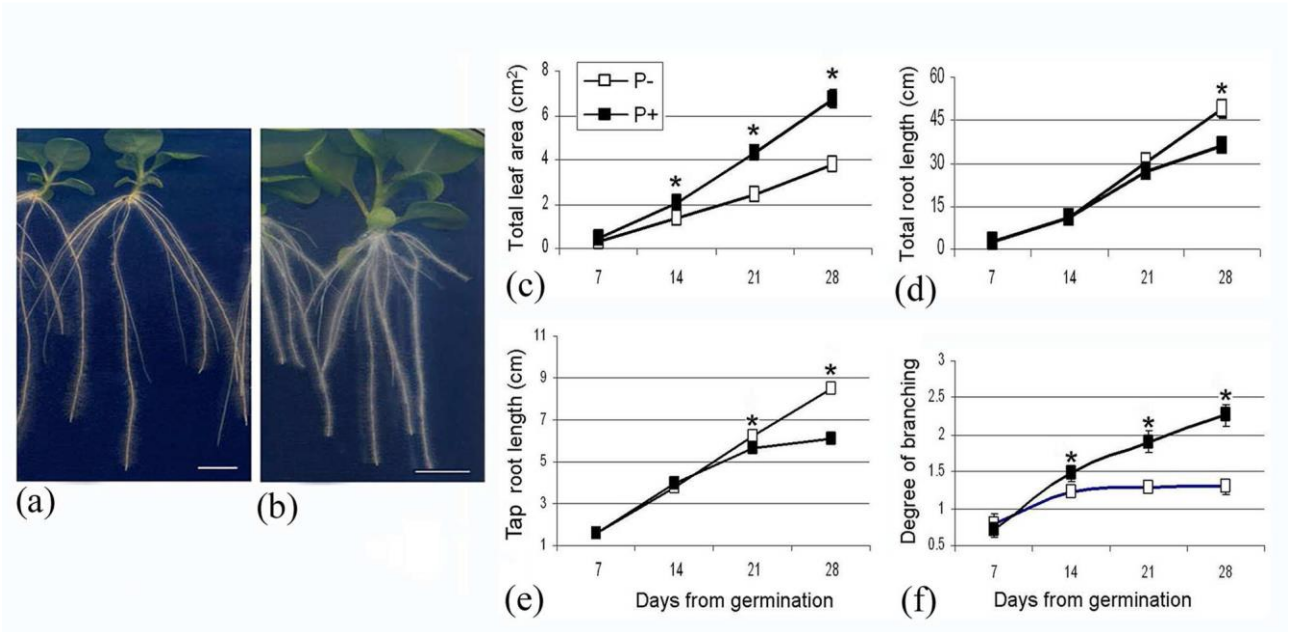


Figure 1. Effects of Pi availability on *Nicotiana tabacum* plant development. (a, b) Scanned tobacco plants grown for 28 days on the surface of agar plates containing low (a) and high (b) Pi media. Bars, 1 cm. (c-f) Morphometric parameters related to leaf and root growth. Average values (±SE) are given for the total leaf area per plant (c), the total (d) and the tap (e) root length, the degree of the tap root branching (f). * indicates significant differences between treatments.

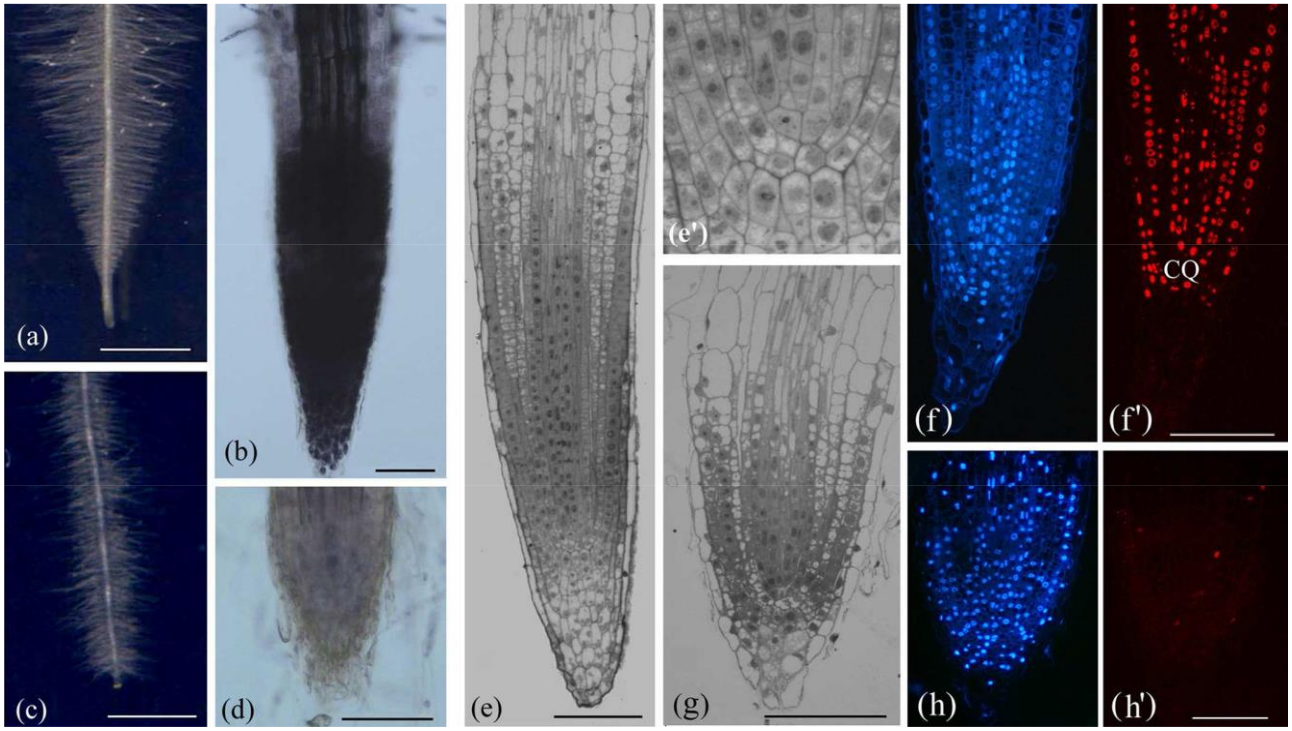

Figure 2. Representative images showing the root tips of the tap roots of *Nicotiana tabacum* plants grown in low (a, b, e-e', f-f') and high (c, d, g, h-h') Pi media, for 28 days from germination. (a, c), Root tips and root hairs of the tap roots. Bars, 1.5 mm. (b, d) Root tips stained with NBT. (e, g) Longitudinal median semi-thin sections (e', magnification of the initial zone of e). (f-f', h-h') Effects of Pi availability on the cell proliferation of the root apices; (f, h), DAPI-stained sections showing all nuclei, (f'-h') indirect immunofluorescence showing the nuclei of proliferating cells (CQ, quiescent centre). (b, d- h') Bars, 100 μm.

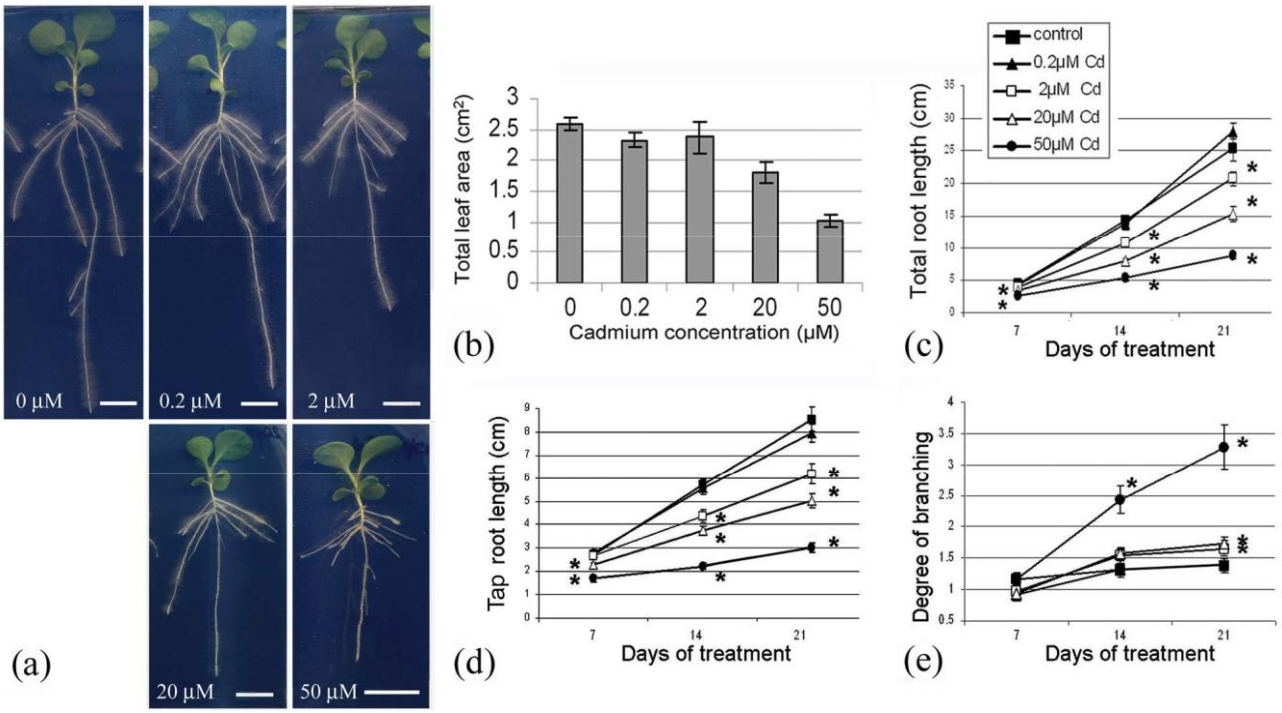


Figure 3. Effects of cadmium treatment on *Nicotiana tabacum* plant development. (a) Scanned tobacco plants grown for 21 days on the surface of agar plates of controls (0 µM) and containing 0.2; 2; 20 and 50 µM $CdCl_2$. Bars, 1 cm. (b-e) morphometric parameters related to leaf and root growth. Average values (±SE) are given for the total leaf area per plant (b), the total (c) and the tap (d) root length, the degree of the tap root branching (e). * indicates significant differences in comparison with controls.