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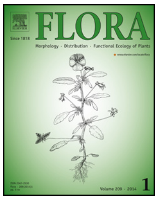

Morphological effects at radicle level by direct contact of invasive *Acacia dealbata* Link

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ABSTRACT

Seeds of *Lactuca sativa* L. (model plant) and *Quillaja saponaria* Mol. (native tree) were subjected to germination in direct contact with leaves, pods and seeds from invasive *Acacia dealbata* Link. (Fabaceae) to reveal whether morphological changes occur at radicle level. Bioassays were carried out with plant material deposited underneath *A. dealbataís* canopy under natural conditions at Mediterranean ecosystem in South America. Segments of radicle tip, cell elongation zone and root hair zone were analyzed under scanning electron microscopy. The three plant parts from *A. dealbata* inhibited the formation of root hairs and altered the rhizodermis (deformation and tissue destruction) of the two recipient species. In the case of *Q. saponaria*, an increase in thickness in the cell elongation zone was observed and internal tissues up to the vascular cylinder in the root hair zone were intensely damaged. The root cap and meristematic region suffered structural changes and damage resulting from the contact with leaves and pods. All morphological effects have been attributed to the impact caused at root level by potential allelochemicals released by different tested plant materials of *A. dealbata*. These results suggest that the morphological changes and damage produced by *A. dealbata* at the radicle level may pose a threat to the growth and survival of the recipient species.

1. Introduction

Acacia dealbata Link (Fabaceae) is one of the most widely distributed species of the genus *Acacia*. Native to Australia (Lorenzo et al., 2010), it is invasive species in Southeastern Europe (Rodríguez-Echeverría et al., 2013), South Africa and South America (Richardson et al., 2011); specifically in Chile (Fuentes-Ramírez et al., 2011), where it was introduced in 1881 (Philippi, 1881; Fuentes et al., 2013) and occupies about 100 000 hectares in the Biobío Region (Pauchard and Maheu-Giroux, 2007). The expansion of *A. dealbata* populations is associated with riparian habitats, roadsides and anthropogenic disturbances (Matthei, 1995; Pauchard and Maheu-Giroux, 2007; Peña et al., 2007). Some characteristics which make this species a successful invader are, for instance; its phenotypic plasticity (Pholman et al., 2005; Aguilera et al., 2015a), its high capacity of vegetative regeneration from rhizomes (Sheppard et al., 2006; González-Muñoz et al., 2011) and its allelopathic properties (Carballeira and Reigosa, 1999; Lorenzo et al., 2008; Lorenzo et al., 2011; Lorenzo et al., 2013). Allelopathy is the effect of one plant on another through the production of chemical compounds that are released into the environment (Rice, 1984; Lambers et al., 2008). In nature, allelopathy can be a result of the joint action of several allelochemicals, which can affect crucial physiological processes in native plants (Inderjit and Duke, 2003).

Several studies show evidences that allelochemicals released by *A. dealbata* has inhibitory effects on the germination of different species (Carballeira and Reigosa, 1999; Lorenzo et al., 2008), also inducing changes in net photosynthesis and the respiration rates of some native understory species in northwestern Spain (Lorenzo et al., 2011). The absence or scarcity of vegetation under the canopy of *A. dealbata* is often attributed to an allelopathy phenomenon (Fuentes-Ramírez et al., 2011) and, consequently, the inhibitory effects to the secondary metabolites released by this species (Lorenzo et al., 2013). Volatile compounds were identified in different *A. dealbata* parts (Souza-Alonso et al., 2014). Until now, Aguilera et al. (2015b) have identified nonvolatile biomolecules present in *A. dealbata* (leaves, flowers, pods and bark) and those involved in this process, attributing great importance to resorcinol, lupanine and stigmasterol, among others. In addition, they detected root growth inhibition in *Lactuca sativa* L., various receiving native herbaceous plants (e.g. *Helenium aromaticum* (Hook.) L.H. Bailey and *Rodophiala maculata* (Líella.) Ravenna) and trees species (e.g. *Quillaja saponaria* Mol.), which produced necrosis and ultimately compromised their survival. Furthermore, these authors suspected

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that the mentioned alterations at radicles may be associated with damage at tissue level.

Therefore, anatomical responses of cells and tissues due to action of water-soluble substances or allelochemicals compounds need to be elucidated and the morphology of seedlings grown in the presence of a phytotoxin may also yield important information (Dayan et al., 2000). Very few microscopic approaches at ultrastructural level have been conducted on allelopathic effects of extracts or allelochemicals (Chon and Nelson, 2000; Graña et al., 2013). At this level, by means of electron microscopy, a study showed that barleyís ~(*Hordeum vulgare*) hordenine and gramine allelochemicals produced damage on cell walls, an increase both in size and number of vacuoles, autophagy, and disorganization of organelles (Liu and Lovett, 1993). More recently, a study on allelopathic interference of benzoic acid against mustard (*Brassica juncea*) seedling growth showed irregular shaped cells arranged in a disorganized manner and cell organelles disruption at cellular level (Kaur et al., 2005). This result indicates that damage to the mustard root at cellular level was clearly shown by the changes in cell morphology and internal organization. So far it has never been verified whether the inhibition caused by allelochemicals released by *A. dealbata* have any involvement at the morphological level. The aim of present work was to determine whether the direct contact with leaves, pods and seeds of *A. dealbata* morphologically alters the rootlets of recipient seedlings of *L. sativa* L. and *Q. saponaria* Mol. In this way, we shall obtain greater understanding of the mechanisms of action from allelochemicals released by this invasive species.

2. Materials and methods

2.1. Plant material

Plant material from *A. dealbata* was collected in the Quillón Commune, approximately 67 km to the north of the city of Concepción in the Biobío Region of Chile (36°50′58.81″ S, 72°32′4.91″ W at 140 m.a.s.l), characterized by its Mediterranean climate. The natural predominant vegetation is a forest dominated by *Q. saponaria*, *Lithrea caustica* (Mol.) Hook. et Arn and *Peumus boldus* Mol., with a rich understory consisting of herbs and leguminous shrubs which is currently invaded by *A. dealbata*. Plant material was collected under *A. dealbata*'s canopy after its natural deposition in 2013; pods and seeds in January and leaves in June. Subsequently, all plant samples were stored in plastic bags under refrigerated conditions (~8 °C) until its use for bioassays. Seeds of *L. sativa* (model plant) were purchased at the Agroflora local market and seeds of *Q. saponaria* (native tree) were collected in San Carlos de Apoquindo (33°27′ S, 70°42′ W at 900 m.a.s.l) at the Mediterranean Ecological Research Station (EDIEM, Pontificia Universidad Católica de Chile), located in the foothills of the Andes. We used a model plant and a native tree to compare morphological alterations at rhizodermis level. Besides, *L. sativa* was chosen as a model species because of its rapid germination and allelopathic sensitivity (Lorenzo et al., 2008; Coelho de Oliveira et al., 2008; Centenaro et al., 2009).

2.2. Bioassay

The amount of plant material per 63.3 cm^2 (area of a Petri dish of 9 cm of diameter) deposited naturally under canopy of *A. dealbata* was calculated randomly using 25 quadrats of equivalent area. Predominant plant material inside these quadrats was collected, weighed and used to quantify a biomass fall rate (expressed in g of litter). Litter averages of the leaves, pods and seeds were 314.4, 518.8 and 676.1 g/m^2, respectively. The equivalent for a Petri dish – 9 cm in diameter – were 2, 3.3 and 4.3 g, respectively. Each different plant material was placed into a Petri dish covered with a Whatman No 1 paper disc and then watered with 20 mL (leaves and pods) or 8 mL (seeds) of distilled water. The volume of water was chosen based on previous experiments (Aguilera, 2015c), in order to ensure the minimum amount that guarantees germination. Petri dishes devoid of plant material and covered with Whatman No 1 paper disc soaked with 5 mL of distilled water were used as controls. Thirty seeds of *L. sativa* or 25 seeds of *Q. saponaria* were sown in each Petri dish, which were sealed with Parafilm® to prevent evaporation and randomly placed in a growth chamber (Bioref-Pitec, Santiago, Chile) at 70–75% relative humidity, 12 h light/dark (~80 μmol m^{-2} s^{-1}) and 20 °C for 7 and 15 days, respectively. Each treatment was replicated seven times. The pH was measured directly in Petri dishes at the beginning and at the end of the experiment by pH-indicator strips pH 0–14 (Acilit® MERCK, Darmstadt, Germany), and ranged from 6 to 6.5, an adequate value for the germination and seedling growth of both species (Lardizábal, 2005; Rodríguez et al., 2005).

2.3. Scanning electron microscopy

Three-millimeter segments of radicle tip, cell elongation zone and root hair zone of each treatment sample as well as complete roots were fixed for 24 h in 2.5% glutaraldehyde in a sodium phosphate buffer pH 7.2 at 4 °C. They were then washed with a sodium phosphate buffer (0.1 M) two times for 10 min and were fixed in osmium tetroxide 1% in a 0.1 M sodium phosphate buffer for 2 h at 4 °C, after they were washed with the same buffer twice during 10 min each time. The cross-sections cuts consisted of thin sections (about 200 μm) and were cut with a rotation microtome HM 550 (MICROM International GmbH, Walldorf). Then, the samples were dehydrated in a 30–100% ethanol series and were dehydrated a second time in liquid CO_2 by a critical point dryer (Balzers Union FL-9496, Holland) (Anderson, 1951). Immediately, cuts and complete roots were mounted on a aluminum sample holder with a carbon film and were then gold plated by a metallizer (Edwards S 150 Sputter Coater, USA) for 3 min at 30 mA, leaving a thickness of approximately 400 Å. Specimens were viewed under scanning electron microscopy (JEOL JSM- 6380 LV, Japan) and a morphological changes comparison in root hairs and tissues (rhizodermis, parenchyma cortex, endoderm and vascular tissue) was done between control and treatments. Widths of five to seven samples were measured from micrographs using the cell elongation zone and measurements were made at 467 μm from the lower end of the radicle tip.

2.4. Statistics

Bioassays were established on basis of a completely randomized experimental design. The data was analyzed by one-way ANOVA using STATISTICA 8.0 for Windows (StatSoft,, 2007). Data normality and variances homogeneity were respectively evaluated with Kolmogorov–Smirnoff and Levene tests. Tukey's test was used to determine differences between means at $P < 0.05$.

3. Results and discussion

Both studied species suffered morphological alterations at radicle level due to direct contact with different parts of *A. dealbata*; particularly, the root hair zone revealed notable damage. In case of *L. sativa*, abundant root hairs were produced by control (Fig 1A), which allows that seedlings could absorb water and grow normally. However, *A. dealbata* pods prevented formation of root hairs and damaged the rhizodermis tissue (Fig. 1B). The effect caused by leaves of *A. dealbata* on radicles of *L. sativa* was similar, although a more intense rhizodermis tissue destruction was detected (Fig 1C.), meanwhile seeds reduced considerably the root hairs formation and large spaces were uncovered at the rhizodermis level (Fig. 1D).

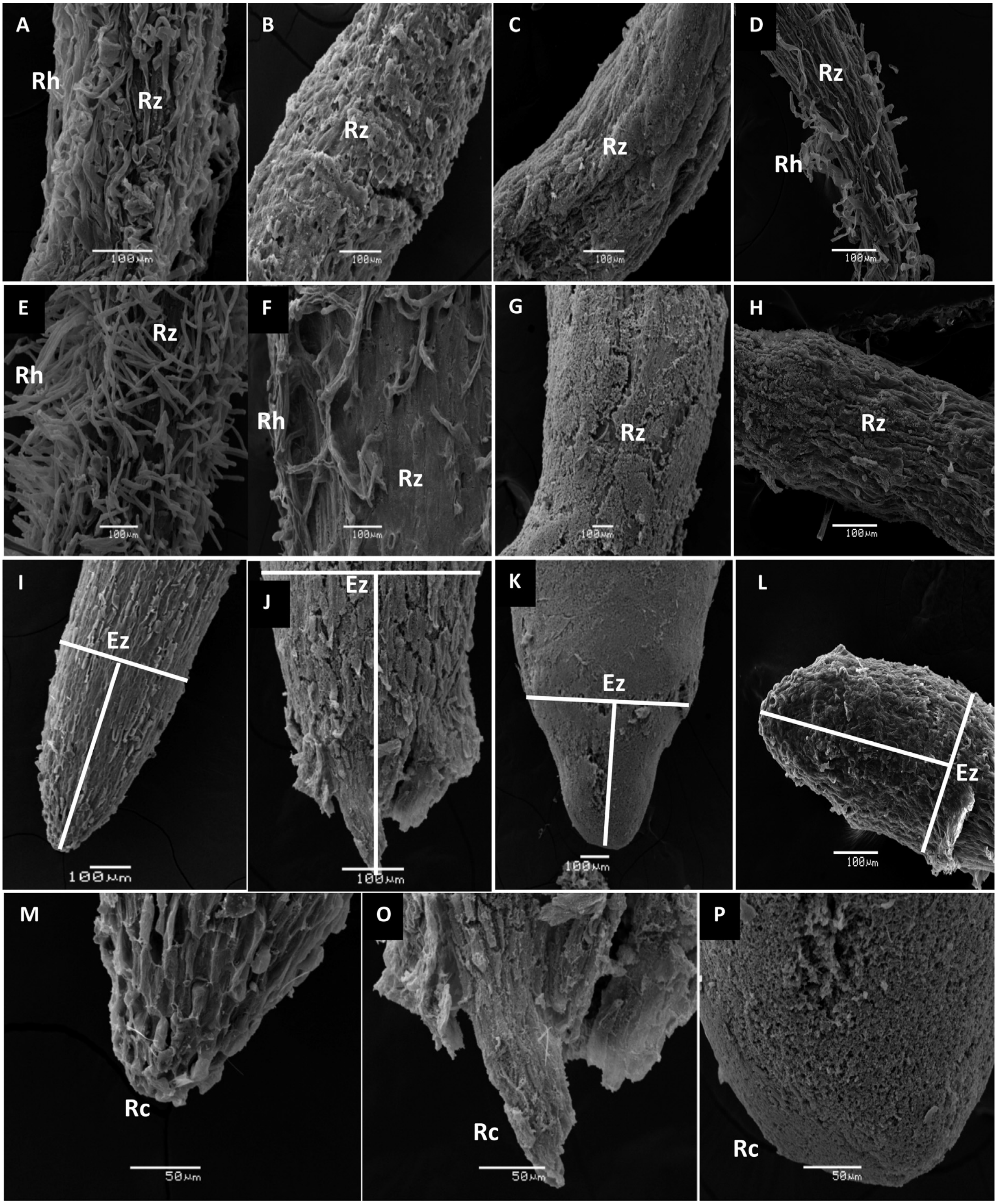

Fig. 1. Direct action of different plant parts of *A. dealbata* on radicle zones of seedlings of *L. sativa* (A–D) and *Q. saponaria* (E–P). A: abundant Rh in control, B: pods inhibited formation of Rh and destroyed Rz, C: leaves inhibited formation of Rh and destroyed intensely Rz, D: seeds induced inhibition and destruction of Rh, E: abundant Rh in control, F: pods induced inhibition and destruction of Rh, G: leaves inhibited formation of Rh and destroyed intensely Rz, H: seeds inhibited formation of Rh and destroyed Rz, I: width of Ez in control, J: width of Ez induced by pods, K: width of Ez induced by leaves, L: width of Ez induced by seeds, M: Rc in control, O: Rc destroyed by action of pods, P: Rc destroyed by action of leaves. Rh: root hairs, Rz: rhizodermis, Ez: cell elongation zone, Rc: root cap. White lines from root tip to Ez are 467 µm long.

The root hair zone of *Q. saponaria* was affected in a very similar way; in this case, control also exhibited abundant and apparent turgor according to visual and micrographs observation (Fig. 1E), while *A. dealbata* pods induced formation of few root hairs, which were noted to be partially destroyed, leaving the rhizodermis almost completely uncovered (Fig. 1F). As occurred with *L. sativa*, the leaves of *A. dealbata* inhibited the formation of root hairs in *Q. saponaria* and caused substantial injury at the rhizodermis level (Fig. 1G). However, this species was much more sensitive to the effect of the *A. dealbata's* seeds, causing an inhibition of root hairs formation (Fig. 1H).

Q. saponaria is a native tree that shares the same distribution range as *A. dealbata* in Chile (Fuentes et al., 2013), therefore it is particular interest in knowing more about possible elements that

Table 1
Effect of different plant parts of A. dealbata on the width of the cell elongation zone (WEZ) from radicles of *Q. saponaria's* seedlings. Values are means ± SD, $n = 7$. Different letters denote significant differences between treatments after one-way ANOVA ($P < 0.05$) and Tukeyís post hoc test.

Treatments	WEZ (μm)
Control	316.3 ± 1.3 d
Pods	334.2 ± 1.8 c
Seeds	403.6 ± 4.5 b
Leaves	551.6 ± 2.3 a

would help predict the effects that it may suffer when struck by the invasion front of *A. dealbata*. For these reasons, cell elongation zone level and root cap were also observed. The cell elongation zone thickness showed a significant increase ($P < 0.05$) induced by the different *A. dealbata* parts (Fig. 1J–L) respect to control (Fig. 1I) (Table 1). The seeds and leaves caused the greatest increase in thickness of this zone; however, in previous studies found that those are the two parts of the invasive plant that most inhibit radicle growth in several native and non-native species (Aguilera, 2015c). The reason for this unbalanced cell growth is uncertain, but it has frequently been observed in relation to the action of several allelochemicals (Burgos et al., 2004). It could be a combined effect of growth retardation induced by auxin (Hoshi et al., 1994; Graña et al., 2013) and destabilization of cell walls due to the increased peroxidase activity of the cell wall in response to phytotoxic compounds (Gonzales and Rojas, 1999) released, in this study, by the plant material of *A. dealbata*. Although meristem and immature cells have small vacuoles, it has been shown that the presence of allelochemicals can induce excessive vacuolization for lytic functions and contribute to the degradation of phytotoxins (Burgos et al., 2004); so radicle parts thickness increase may be an indication of the occurrence of mentioned phenomenon.

The rhizodermis tissue in the root cap was seen to be organized and well structured (Fig. 1M) in control; but, by contrast and in spite of showing different expressions of damage, the first bands of the root cap cells were destroyed by pods (Fig. 1O) and leaves (Fig. 1P). Also, columella cells containing amyloplasts responsible for gravity sensing were affected, the columella can also respond to light as well as pressure of soil particles (Burgos et al., 2004). This damage at the root cap level indicates that when no such cells are formed or destroyed, the mucilaginous gel responsible for preventing dehydration and facilitating penetration of the roots in the ground, amongst other functions, will not form (Taiz and Zeiger, 2010). Since the meristematic zone is just below the root cap, it was also damaged, while the apical meristem was left unprotected and subject to mechanical injury occurring during movement through the soil's profile under natural conditions. The meristematic region in several species, e.g., *Arabidopsis thaliana*, is about a quarter of a millimeter long (Taiz and Zeiger, 2010) and the two layers of cells closest to the apex of the root forms in the dicots (Barlow et al., 1984). As such, serious affectations in the root cap with meristem damage interfere with cell division and jeopardize radicle growth. This is consistent with the inhibition that was detected in radicle growth of *L. sativa* and the native species *Q. saponaria*, *H. aromaticum* and *Rhodophiala maculata* seedlings when subjected to direct contact and aqueous extracts from different parts of *A. dealbata* (Aguilera et al., 2015c).

Observations on the cross-sections cuts at the root hair level zone in *Q. saponaria* showed normal structures of cells and tissues in control (Fig. 2A). However, the micrographs indicated that pods of *A. dealbata* inhibited the root hairs formation, altered the isodi-

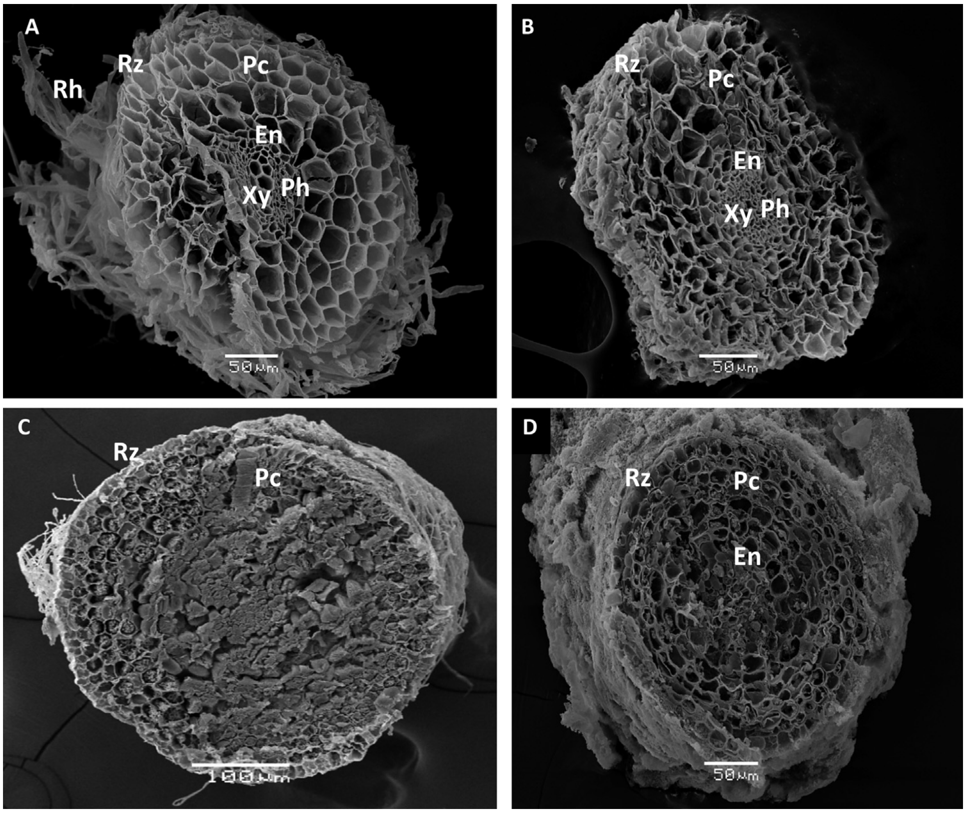

Fig. 2. Direct effect of different plant parts of *A. dealbata* on a root hair zone (cross-section) from radicles of *Q. saponariaís* seedlings. A: Control – normal cell and tissue structures, B: pods induced inhibition of Rh formation and damaged the polyhedral and isodiametric structure of parenchyma cells and turgor of Pc was lost, C: leaves induced inhibition of Rh formation, caused compaction at cortex level and the tissue differentiation and vascular connection were lost, D: seeds induced inhibition of Rh formation, deformation at cortex level and vascular connection was lost. Rh: root hairs, Rz: rhizodermis, Pc: parenchyma cortex, En: endoderm, Ph: phloem, Xy: xylem.

ametric and polyhedral structure of parenchymal cells, and caused a loss of turgor (Fig. 2B). Intense damage provoked by the leaves reached the internal tissues of the radicle as well as its rhizodermis, expressing itself as an amorphous and compact structure where cells and tissues formed a caking effect that also affects the vascular connection (Fig. 2C). Simultaneously, the damages induced by *A. dealbata* seeds were less intense and concentrated mainly in the rhizodermis, although cortex deformations were noted, and xylem and phloem were not clearly distinguished (Fig. 2D). Similar disorganization was observed in the *A. thaliana* meristems which had been treated with the allelochemical citral (Graña et al., 2013). Interestingly, it is reported the expansion of the vascular cylinder and of the layers of the cortex cells may lead to abnormalities such as observed here (Romero-Romero et al., 2005; Mojarad et al., 2013).

In general, morphological changes and damage are intimately related (Lambers et al., 2008). Morphological damage is considered as the most intense expression of morphological changes in a short time. In this regard, here, morphological changes were associated with decrease of root hairs, width variation of the cell elongation zone, alteration of the polyhedral structure of parenchyma cells, and a poor definition of vascular tissues. At the same time, morphological damage pointed to cellular and tissue destruction, inhibition in root hairs formation, undifferentiation of root tissues, and inability of the vascular connection. The major change found in the treated roots could be due to auxin content that play an important role in the absence of root hairs, as root hair formation (Graña et al., 2013). Presence of some environmental factors, auxin and expansins are required for growth and development of root hairs (Schiefelbein, 2000). The manifestation of these phenomena seems due to the potential presence of allelochemicals in the growing medium which in turn suggests that the plant tried to respond to chemical stress at an early stage of growth. However, the ability of the recipient plants to survive a constant exposure to chemicals released by *A. dealbata* appears limited. In this context, previous studies have shown that some allelochemicals alter cell membranes by disrupting permeability, ions flow and hydraulic activity at root level, resulting in cascading effects that cause severe damage to the stomatal function and the photosynthesis and respiration rates (Einhellig, 2004). Few morphological studies have been devoted to evaluating plant responses to stress by allelochemicals. Despite this, a number of published works on other species are in agreement with the results presented here (e.g., Aliotta et al., 1994; Gasparikova et al., 1996; Cruz-Ortega et al., 1998; Burgos et al., 2004; Romero-Romero et al., 2005; Mojarad et al., 2013).

In summary, the plant material from *A. dealbata* caused changes and structural damage in *L. sativa* and *Q. saponaria* radicles. The three plant parts inhibited the root hairs formation and altered rhizodermis; however, leaves induced alterations in vascular cylinder of *Q. saponaria*, while the root cap was dismantled by action of pods and leaves. The effects on thickening, cell organization and the absence of root hairs suggest that *A. dealbata* litter act as releaser of powerful phytotoxic compounds. These morphological changes or damage are potential risks for the growth and survival of the recipient species due to limitations to absorb water and nutrients, as well as the photosynthetic effects associated. This study contributes to understanding the modes of action of allelochemicals released by *A. dealbata* in Mediterranean ecosystems of South America, which might be similar in other Mediterranean ecosystems.

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