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Allelopathic effects of aqueous extracts of *Artistolochia esperanzae* O.Kuntze on development of *Sesamum indicum* L. seedlings

Ana Beatriz Gatti[1], Alfredo Gui Ferreira[2,4], Marcos Arduin[3] e Sonia Cristina Gualtieri de Andrade Perez[3]

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RESUMO – (Efeitos alelopáticos de extratos aquosos de *Aristolochia esperanzae* O.Kuntze sobre desenvolvimento de plântulas de *Sesumum indicum* L.). *Aristolochia esperanzae* é uma trepadeira que ocorre no cerrado do sudeste do Brasil. Os objetivos deste trabalho foram de identificar os efeitos dos extratos aquosos de *A. esperanzae* sobre a germinação, crescimento da raiz e de células do xilema de plântulas de gergelim. Extratos de folhas, caule e raiz foram preparados nas concentrações de 1,5 e 3,0%. Os extratos causaram alterações na germinação e no crescimento das plântulas com inibição maior causada pelos extratos de raízes. Observou-se mudanças morfológicas e decréscimo no crescimento e desenvolvimento das plântulas de gergelim. Os extratos de *A. esperanzae* causaram uma inibição de até 50% no tamanho das células do xilema das raízes e mudanças na raiz primária e no número de raízes secundárias.
Palavras-chave: Papo-de-perú, alelopatia, crescimento de raiz, desenvolvimento de xilema, cerrado

ABSTRACT – (Allelopathic effects of aqueous extracts of *Aristolochia esperanzae* O.Kuntze on development of *Sesamum indicum* L. seedlings). *Aristolochia esperanzae* is a climbing plant that occurs in the savanna regions of Brazil. The aim of this work was to identify the effects of aqueous extracts of *A. esperanzae* on germination, root growth and xylem cell development of sesame seedlings. Leaf and shoot extracts were prepared at concentrations of 1.5 and 3%. Extracts caused marked changes in germination and seedling growth with greatest inhibition produced by root extracts. Morphological changes and decreased growth and development of seedlings were also observed. The extracts of *A. esperanzae* caused a reduction of 50% in the size of root xylem cells and marked changes in the primary root and in the number of secondary roots.
Key words: "Papo-de-perú", allelopathy, root growth, xylem development, savanna

Introduction

Allelopathy is defined as the beneficial or harmful influence of chemical substances released by plants that can alter the growth and development of nearby plants or microorganisms. Allelochemicals may be present in all plant organs, including leaves, flowers, fruits, roots, rhizomes, stems and seeds (Putnam & Tang 1986), some of which can store these compounds. However, the quantity and emission pathway varies from species to species (Friedman 1995).

Typical allelopathic inhibitory effects result from the action of groups of allelochemicals that collectively interfere in various physiological processes altering the growth patterns of plants (Einhellig, 1996; Parvez *et al*. 2004; Kil & Shim 2006). In most cases the organic compounds that are inhibiting at higher concentration and they are stimulating at smaller ones (Rice 1984). The action of allelochemicals can affect the respiration, photosynthesis, enzyme activity, water relations, stomatal opening, hormone levels, mineral availability, cell division and elongation, and structure and permeability of cell membranes and walls (Chou 1999; Reigosa *et al*. 1999).

Many studies have found that roots are more sensitive to allelochemicals than aerial parts of seedlings (Bagchi *et al.* 1997; Hamdi *et al.* 2001; Parvez *et al*. 2003; Rahman 2006; Punjani *et al*. 2006; Oliveira & Campos 2006; Ercoli *et al*. 2007). The inhibition of root growth and development by allelochemicals can be due to changes in DNA synthesis in cells of apical root meristem, alteration of the mitochondrial metabolism (Abrahim *et al*. 2000) or changes in cell mitotic indices (Dayan *et al*. 1999; Romagni *et al*. 2000; Pires *et al*. 2001; Iganci *et al*. 2006).

Kaur *et al*. (2005) demonstrated that benzoic acid produces irregularities in mustard root cells, which were disorganized, inhibiting root growth. Cells at the root tips of *Phaseolus vulgaris* also were stunted and compacted when the seedlings of this species were grown under the influence of aqueous extracts of *Sicyos deppei* (Cruz-Ortega *et al.* 1998). Morphological changes are signs of previous changes that occur at the cellular and molecular level (Ferreira & Áquila 2000). Alterations in the cell membranes can be considered one of the first effects caused by allelochemicals, and these effects may then trigger secondary effects (Barkosky *et al*. 2000).

Aristolochia esperanzae, known popularly in Brazil as *papo-de-peru* (turkey crop) and *mil-homens* (thousand-men), among other names, is a pioneer species and is considered the most frequent among members of the genus in the savanna regions of the state of São Paulo (Cappelari 1991). Various works have reported the presence of terpenes, diterpenes, lignans and aristolochic *acid* in *Aristolochia* species, including *A. esperanzae* (Priestap *et al.* 1971; Lopes *et al*. 1988 and Lopes & Bolzani 1988). Stem and root extracts of *A. esperanzae* caused abnormalities and inhibited root growth of *Lactuca sativa* and *Raphanus sativus* seedlings (Gatti *et al.* 2004). By releasing allelochemicals, this species can influence the succession of plants in savanna grasslands because it is a pioneer species and widely distributed in the environment.

[1] São Carlos Federal University, Graduate Program in Ecology and Natural Resources. São Carlos, SP, Brazil
[2] Rio Grande do Sul Federal University, Biosciences Institute, Botany Department. Porto Alegre, RS, Brazil
[3] São Carlos Federal University, Center for Biological and Health Sciences, Botany Department. São Carlos, SP, Brazil
[4] Author for correspondence: ferreira@unb.br

Most studies on allelopathy are focused on invasive species and plants of agricultural interest, or on identifying and isolating chemical substances with potential use as herbicides. Little is known of allelopathic interference in biomes such as savannas, which have been suffering rapid degradation. Besides this, the succession of species can be affected by allelopathy, permitting pioneer species to establish themselves thanks to the release of allelochemicals (Reigosa *et al*. 1999). However, allelochemicals can be selective in their actions and plants can be selective in their responses. For this reason, it is difficult to determine the action of these compounds (Seigler, 1996). Many studies have shown changes in germination and growth of target seedlings, but few have evidenced the physiology and method of action of allelochemicals (Reigosa *et al*. 1999; Inderjit & Duke, 2003). In this study sesame seeds were used since they have very uniform germination and seedling development.

Therefore, the aim of this study was to examine the effects of leaf, stem and root aqueous extracts of *Aristolochia esperanzae* to identify effects on the germination of sesame seeds and if they alter the morphology and anatomy of the target seedlings.

Material and methods

Plant material – Leaves, stems and roots of *Aristolochia esperanzae* O.Kuntze (Aristolochiaceae) were collected on the campus of São Carlos Federal University (São Paulo, Brazil) in April 2005. The material was kept frozen until preparation of the extracts**.**

Aqueous extracts – The plant material was weighed and ground with distilled water in an industrial blender and the homogenate was left to settle for three hours in the dark. Then each extract was filtered using a vacuum pump coupled to a Büchner funnel covered with qualitative filter paper and immediately utilized. The extracts were prepared at concentrations of 1.5 and 3.0% (1.5g/100 mL and 3.0 g/100 mL) of dry material, at which time the percentage of water in these materials was obtained. The target species used in the bioassays was sesame (*Sesamum indicum* L., Pedaliaceae) and the effects of the leaf, stem and root extracts of *Aristolochia esperanzae* were compared to those of the control group (grown in distilled water).

Germination bioassay – Thirty sesame seeds were placed in Petri dishes (9 cm diameter) containing a double layer of filter paper moistened with 5 mL of *A. esperanzae* extracts or distilled water, on which 30 sesame seeds were placed. The plates with the seeds were kept in BOD chambers at 28ºC (± 2) and 12-h. photoperiod. The germinated seeds were counted at 12-h intervals during the first seven days and every 24 h thereafter until ten days had elapsed. Those seeds that had emerged a 2 mm radicle were considered germinated. The parameters analyzed were percentage and speed of germination (Labouriau 1983; Borghetti & Ferreira 2004). The experimental setup was totally random with four repetitions for each treatment.

Growth bioassay – Clear plastic boxes (11 x 29 x 9.5 cm) were lined with two sheets of filter paper moistened with 40 mL of extract (or water), covered and placed in clear plastic sacks. Thirty sesame seeds germinated in water (2- to 4-mm-long rootlet) were distributed in the plastic boxes and kept in the climate-controlled chamber under the same conditions as for the germination test. After four days the length of the aerial part and primary root was measured with a digital caliper.

Examination of xylem elements – For this evaluation the seedlings were grown in the same temperature and light conditions as in the other bioassays. After four days, the seedlings were removed from the boxes, the primary root segment was detached and immersed in 70% ethanol.

The modified Fuchs method of staining was used (Kraus & Arduin, 1997), where the roots remained immersed in alcohol (70%) for seven days and then were placed in a solution of 25% NaOH for 24 to 48 h until the material was clarified. Then the root segments were immersed in a 2% solution of glacial acetic acid for 30 min., 30% ethanol for 5 min., following by safranin in a water-alcohol medium (50%) for 30 min., then a solution of 30% ethanol + 0.5 mL of acetic acid for 5 min., and finally 30% ethanol for 5 min.. After staining, the material was mounted on glass slides with the roots in Apathy's syrup (Kraus & Arduin 1997) for observation under an optical microscope (Olympus–BX41) coupled to a camera (Sony CCD-IRIS). Four primary roots were used from sesame seedlings grown in water or the different extracts of *Aristolochia esperanzae*. One-half of the length of each root was photographed, from the central region upward. From each photograph ten central cells of the metaxylem with 20X magnification (Image Pro Plus program) were measured.

Data treatment and statistical analysis – The experimental setup and bioassays were totally random, with four replicates for each treatment. The percentage values were transformed into arc sin ($\sqrt{\%}$). The Kolmogorov-Smirnov (Lillifors) test for normality was applied to all the groups of values obtained (treatments). The data were submitted to variance analysis (single criterion), and depending on the distribution, the Kruskal-Wallis (non-parametric) or Tukey (parametric) test was used at 5% significance (Santana & Ranal 2005). The statistical analyses were run in the BioEstat 3.0 program.

Results and discussion

Germination bioassay – The leaf and stem extracts did not cause any changes in the germination percentage of the sesame seeds at any of the concentrations tested. However, the 3.0% root extract caused a significant reduction in the germination percentage. Regarding germination speed, all the extracts caused a delay in germination except for the 1.5% stem extract (Fig. 1).

Alterations in germination patterns can be caused by changes in the permeability of cell membranes, transcription and translation of RNA, integrity of secondary messengers, respiration, conformation of enzymes and receptors, or a combination of these changes (Rizvi & Rizvi 1992; Ferreira & Áquila 2000). For example, 6-methoxy-2-benzoxalinone (MBOA) inhibits the germination of lettuce seeds by impeding inducement of α-amylase synthesis, which mobilizes the stored reserves and maintains seed respiratory activity (Kato-Noguchi & Macias, 2005). Baleroni *et al.* (2000) showed that *p*-coumaric and ferulic acids increased total lipid content in the cotyledons of canola seeds and suggested that this change is due to reduced mobilization of reserves during germination in the presence of these phenolic compounds.

Often the allelopathic effect is not observed in the final germination percentage, but rather in the speed of germination, which can provide important indications of the allelochemical (Ferreira 2004). Delays in seed germination of any species can have important biological implications, because this will affect the establishment of seedlings in natural conditions (Escudero 2000; Chaves *et al*. 2001) and their chances of competing for resources with neighboring species (Xingxinag *et al.* 2009).

Aristolochia esperanzae extracts altered the germination process of the sesame seeds, demonstrating that these changes can also occur in nature. Among the extracts from the different organs used, those from the root inhibited germination the most. This effect varied depending on

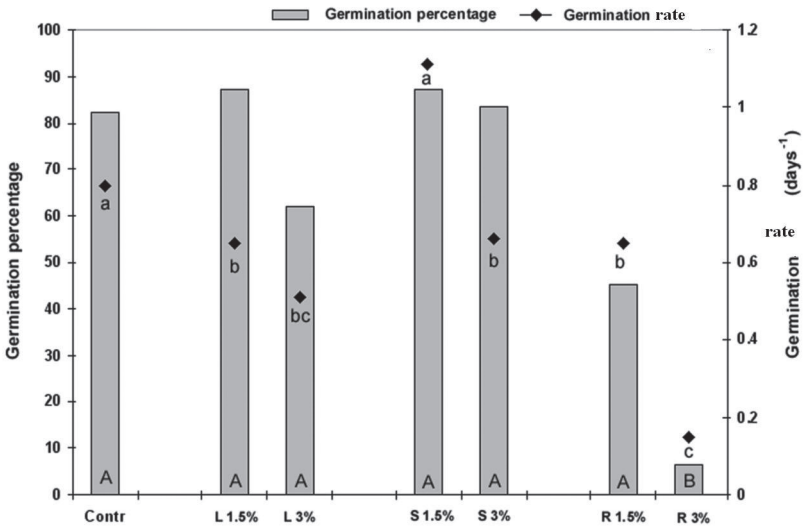

Figure 1. Germination percentage and speed of sesame seeds submitted to aqueous extracts of leaves (L 1.5% and L 3%), stems (S 1.5% and S 3%) and roots (R 1.5% and R 3%) of *Aristolochia esperanzae*. Means followed by the same capital letters did not differ according to the Kruskal-Wallis test for germination percentage, and those followed by lowercase letters did not differ according to the Tukey test for germination speed.

the concentrations used, with total suppression at 3% concentration (Fig. 1).

Growth bioassay – Growth of the shoot was stimulated when the sesame seedlings were grown in the presence of leaf and stem extracts (1.5 and 3.0%), but in the presence of the root extracts (1.5 and 3.0%) it did not differ statistically from the control group (Fig. 2 – top). Other studies have also identified stimulated plant growth in the presence of extracts. Extracts of *Euphorbia serpens* stimulated growth of the aerial parts and roots of *Lactuca sativa* (Dana & Domingo 2006), and leaf extracts of *Phytolacca americana* stimulated growth of the aerial parts and roots of *Cassia mimosoides* (Kim *et al*. 2005).

The leaf extract had no significant effect on growth of the primary root. However, the stem and root extracts caused a significant reduction in growth. They were darkened and rotted when kept in the 3.0% root extract. These results reveal that regarding initial growth, the primary root of the sesame seedlings was more sensitive to the root extracts of *Aristolochia esperanzae* than was the aerial part (Fig. 2). Others authors have also reported that roots are more sensitive to allelochemicals than aerial parts (Hamdi *et al.* 2001; Parvez *et al.* 2003; Punjani *et al*. 2006; Abdelgaleil & Hashinaga, 2007; Ercoli *et al*. 2007). Extracts of alfalfa (*Medicago sativa*) and coumarin increased the diameter of alfalfa roots (Chon *et al.* 2002). According to the authors, this was due to the expansion of the central vascular cylinder and changes in cortex cell layers. In another study, the monoterpenes camphor, eucalyptol, limonene and α-pinene inhibited the growth of corn roots, and the authors demonstrated that this happened because of changes in the mitochondrial metabolism, thus altering various other physiological and metabolic processes associated with growth and development of the plants (Abrahim *et al*. 2000). Corn seedlings showed a reduced mitotic index in the presence of *Leucena leucocephala* mulch and the authors observed that the absence of cell division and root thickening was due to the increased activity of the enzyme peroxidase in these seedlings (Pires *et al*. 2001). Other authors have also reported changes in mitotic indices in the presence of allelopathic substances (Dayan *et al*. 1999; Jacobi & Fleck 2000; Pires *et al*. 2001; Iganci *et al*. 2006). Concentrations of 0.1 and 0.15mM of sorgoleone provoked changes in the formation of cell walls and caused deformation of vessel elements, besides discontinuity in the starch sheath (Hallak *et al*. 1999).

In line with these findings and similar to the results of the germination bioassay, the root extracts of *A. esperanzae* had the greatest inhibiting effect, causing morphological changes and reduced growth of the seedlings. Root exudates and residues are commonly known as the two main sources of allelochemicals released into the soil (Yu *et al*. 2000). These compounds are generally stored in root cells for subsequent release (Rice 1984). This is the probable route by which the allelochemicals of *A. esperanzae* are released into the soil, explaining the greater inhibitory activity (both on germination and growth) of the root extracts.

Xylem elements - The average size of the metaxylem cells of the seedlings grown in water was 150.89 µm (±54.11). Those grown in the leaf, stem and root extracts of *Aristolochia esperanzae* had statistically smaller average of metaxylem cell sizes than the control group (around 50% smaller) (Fig. 3 - 4). The data on the percentage of cells distributed in size classes showed that the control group had homogenous cell size distribution, with the highest percentage (37.70%) found for cells between 101-150 µm long. There were no cells lower than 50 µm in the control

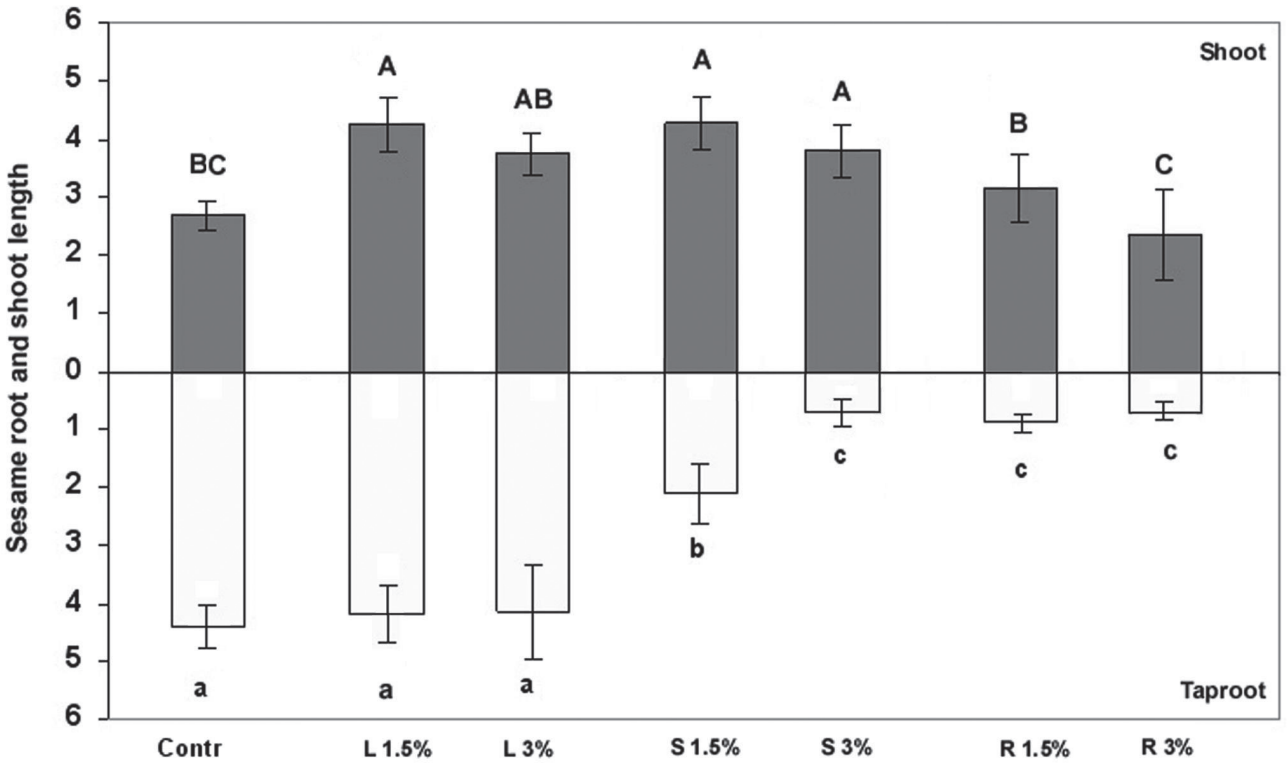

Figure 2. Length of the aerial part and primary root (cm) of sesame seedlings grown under aqueous extracts of leaves (L 1.5% and L 3%), stems (S 1.5% and S 3%) and roots (R 1.5% and R 3%) of *Aristolochia esperanzae*, and the control in water (Contr). Means followed by the same capital letters (aerial part) and lowercase letters (primary root) did not differ according to the Tukey test. (N = 16 ± SD).

group, but there were cells longer than 151 μm. In contrast to the control group, the cells of seedlings grown under the influence of the extracts were more homogeneously distributed regarding size. The cells of seedlings grown with the leaf and stem extracts were predominantly (60%) between 51-100 μm. For the root extracts (1.5 and 3.0%), the majority of cells were also in this size interval (78.8 and 79.5%, respectively). None of the cells from plants grown under the influence of the root extracts were longer than 151 μm (Fig. 5).

A. esperanzae extracts inhibited the growth of sesame roots as was shown above. This indicates the probable interference of allelochemicals present in the extracts with concentrations of different categories of hormones like auxins and cytokinins

Aliotta *et al.* (2004) demonstrated that the expansion of root cells was reduced in the presence of different concentrations of parts of *Olea europea* and this reduction resulted in thickening of the root tip in comparison to the control. According to Al-Wakeel *et al*. (2007), inhibition of cell elongation can be related to the direct action of allelochemicals, by interfering in the process of cell division and thus altering the balance of the different hormones. In the present study, sesame plants grown under the influence of extracts in general showed stunted primary root growth and reduction of 50% in the size of root xylem cells.

The results obtained in this study show that different extracts of *A. esperanzae* caused changes in germination and growth of sesame seedlings. Among the extracts of the different organs utilized, those from the roots had the strongest inhibitory effect on germination and growth, and this inhibition depended on the concentration used, causing morphological changes and diminished growth and development of the seedlings, with total suppression of germination and growth at 3% concentration. Exudation from the roots can be the way the allelochemicals of *A. esperanzae* are released into the soil, explaining the greater inhibitory effect with use of the root extracts. Moreover, the extracts altered the number and size of the lateral roots. Therefore, it is not possible to determine the principal action or direct action of the allelochemicals present in the *A. esperanzae* extracts. However, it is probable they are related to the biosynthetic metabolism, concentration and/or sensitivity of the various plant hormones. Chemical studies are under way to analyze the constituents of the extracts to enable determination of which substances function as allelochemicals.

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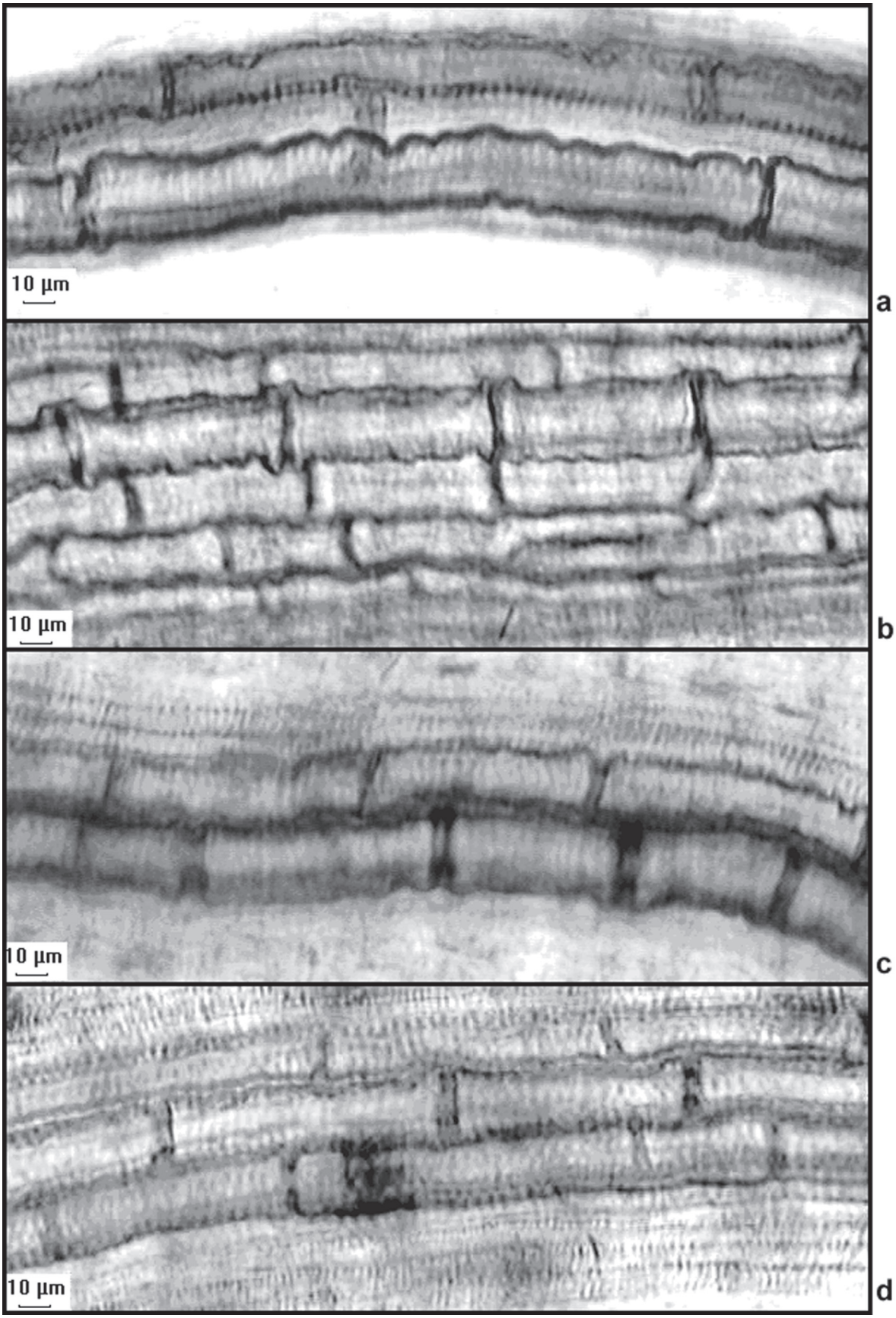

Figure 3. Photomicrographs of root metaxylem cells of sesame seedlings grown in water (A), and in the presence of 3% leaf extract (B), 3% stem extract (C) and 3% root extract (D) of *Aristolochia esperanzae.* (Scale = 10µm)

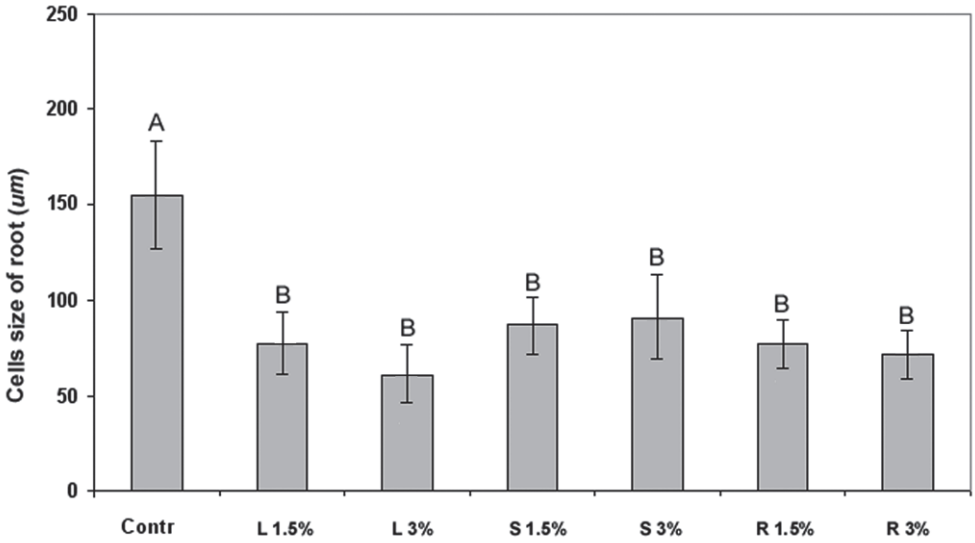

Figure 4. Size of root metaxylem cells of sesame seedlings grown in extracts of leaves (L 1.5% and L 3%), stems (S 1.5% and S 3%) and roots (R 1.5% and R 3%) of *Aristolochia esperanzae*, and the control in water (Contr). Means followed by same letters did not differ according to the Tukey test (N= 16 ± SD).

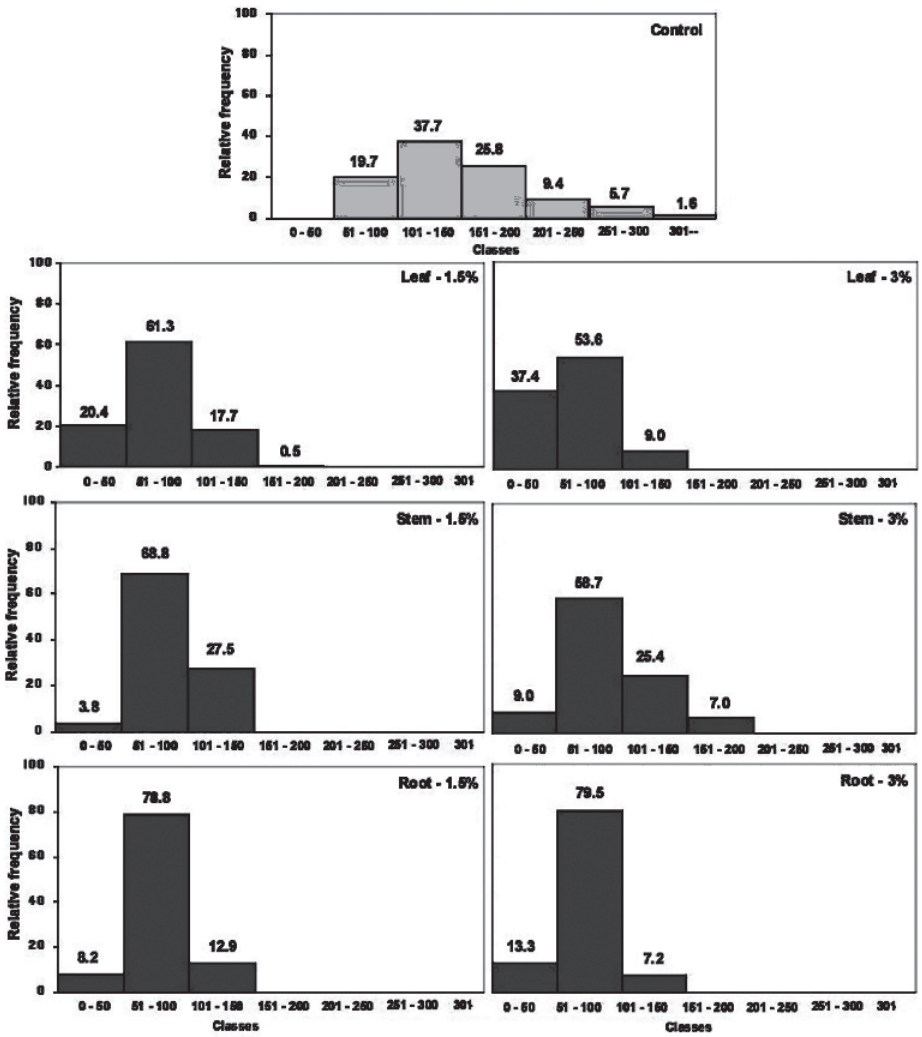

Figure 5 Size-class (µm) of relative frequency of sesame seedling root xylem elements when grown in water (A – control) and in different extracts of leaves (1.5 and 3%), stems (1.5 and 3%) and roots (1.5 and 3%) of *Aristolochia esperanzae*.

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