Leaf anatomy changes related to cultivate *in vivo* and *in vitro* and during pre-acclimatization of *Crambe abyssinica* Hochst

Alterações na anatomia foliar relacionada ao cultivo *in vivo* e *in vitro* e durante a pré-aclimatização de *Crambe abyssinica* Hochst

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ABSTRACT

This study aimed to characterize and evaluate possible modifications in the leaf anatomy of crambe in the *in vivo* and *in vitro* environment and during pre-acclimatization. The results from the comparison of leaves *in vivo*, *in vitro* and during pre-acclimatization indicated that there was no statistical difference with regards to blade, mesophyll, palisade and spongy parenchyma, and midrib. Significant difference existed in the thickness adaxial and abaxial epidermal surfaces, with the highest values being observed for *in vivo* conditions. The area of the vascular bundle was superior and significantly different under *in vitro* conditions. Therefore, the alterations observed in the structure and thickness of the leaves in the pre-acclimatization treatments hampered the process of plantlet establishment *ex vitro*.

Index terms: Oilseeds; micropropagation; mesophyll; leaf histology; plant anatomy.

RESUMO

Este estudo objetivou caracterizar e avaliar possíveis modificações na anatomia das folhas de crambe no ambiente *in vivo*, *in vitro* e durante a pré-aclimatização. Os resultados da comparação das folhas *in vivo, in vitro* e durante a pré-aclimatização, indicou que não houve diferença estatística quanto à espessura do limbo, mesofilo, parênquima paliçádico e esponjoso, e nervura central. Houve diferença significativa na espessura da epiderme nas faces adaxial e abaxial, sendo os valores superiores encontrados na condição *in vivo*. A área do feixe vascular foi superior e significativamente diferente na condição *in vitro*. Portanto, as alterações observadas na estrutura e espessura das folhas dos tratamentos de pré-aclimatização prejudicaram o processo de estabelecimento da plântula *ex vitro*.

Termos para indexação: Oleaginosa; micropropagação; mesofilo; histologia foliar; anatomia vegetal.

INTRODUCTION

Crambe abyssinica is a plant belonging to the family Brassicaceae, being the most economically important species of this genus, with 34 representatives (Wang et al., 2004). Its seeds contain 45-50% of crude protein and up to 35% of oil, which consists to 55-60% of erucic acid (Palmer; Keller, 2011). Such characteristics make it highly valuable for animal feed, industrial and pharmaceutical applications, biodiesel production, as well as fabrication of coatings, detergents, gliding products, cosmetics, lubricants and nylon (Vargas-Lopez et al., 1999; Capelle; Tittonel, 1999; Palmer; Keller, 2011).

The genetic breeding of species like *C. abyssinica*, with the aim of increasing their oil production or altering any compound of interest, has been often employed (Wang; Peng, 1998; Murphy, 1999; Wang; Sonntag; Rudloff, 2003; Wang et al., 2004; Li et al., 2010). However, in order for the genetic breeding to be successful, biotechnological steps beforehand must be efficiently concluded. For crambe, the lack of protocols suitable for micropropagation and the need for plant material in great amount have hampered the development of transgenic plants (Gao et al., 1998; Li et al., 2010), mainly due to the low survival at the final phase – acclimatization (Preece; Sutter, 1991).

The acclimatization process, i.e., the transfer of a plant from the *in vitro* to the *ex vitro* environment, is often a critical step for survival of the plantlet (Apóstolo;

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Brutti; Llorente, 2005). There are great differences between the *in vitro* and the *ex vitro* environments, such as illumination (intensity and quality), relative humidity, nutrients, substrates and gas concentration (Seelye; Burge; Morgan, 2003). The *in vitro* environment offers low irradiance, high humidity, presence of sugar and growth regulators in the substrate, and low gas exchange, with lack of CO_2 and excess of ethylene (Kadlecek et al., 2001). These factors induce disruptions in the growth, development and morphology, involving anatomic and physiological alterations in the plant (Kozai, 1991).

The main physiological disturbance when plantlets are subjected to the *ex vitro* environment is related to excessive loss of water due to inadequate functioning of the stomata, lack of wax and cuticle on the leaf, and a non-functioning root system (Zobayed; Armstrong; Armstrong, 2001; Apóstolo; Brutti; Llorente, 2005; Mathur et al., 2008; Chandra et al., 2010). The anatomic abnormalities may be the result of excess of growth regulators present in the culture medium (Hronkova et al., 2003).

Despite the numerous investigations comparing the structural characteristics of plants *in vitro* and *in vivo* (Dousseau et al., 2008; Mayer et al., 2008; Fermino Junior; Scherwinski-Pereira, 2009; Braga et al., 2011), only few studies exist regarding these characteristics during the process of shoot induction in the successive phases of micropropagation (Appezzato-da-Glória; Vieira; Dornelas, 1999; Apóstolo; Brutti; Llorente, 2005; Hazarika, 2006).

Moreover, few micropropagation protocols mention the steps for acclimatization of crambe (Li et al., 2010, 2011; Chhikara et al., 2012), offering incomplete descriptions and not allowing their repetition. Palmer and Keller (2011) are the only authors relating 70% survival in this phase; however, the authors did not disclose the employed methodology. There are no anatomic studies of *C. abyssinica* available in the specific literature yet.

In this sense, the present study aimed to characterize and evaluate possible modifications in the leaf anatomy of *C. abyssinica* in *in vivo* and *in vitro* cultivate and during pre-acclimatization.

MATERIAL AND METHODS

The experiments were executed at the Laboratory of Plant Tissue Culture at the Center of Agronomic Sciences, and at the Laboratory of Plant Anatomy at the Department of Biological Sciences, both at the Federal University of Espírito Santo, ES – Brazil. Seeds of *C. abyssinica* Hochst cv. FMS brilliant, were used, supplied by the Fundação MS, located in Maracaju, Mato Grosso do Sul – Brazil.

The anatomic characteristics of crambe leaves were evaluated in plantlets cultivated *in vivo*, *in vitro* and during pre-acclimatization. The experiment was carried out in a completely randomized design (CRD) with four treatments, constituted by leaves of plants *in vivo, in vitro* and in pre-acclimatization 01 and 02, with five replicates, each consisting of one leaf.

The plantlets of the treatment *in vivo* were produced by germination of seeds in pots containing dystrophic red-yellow latosol corrected with limestone, which were maintained in greenhouse at a mean temperature of 29 °C. After 30 days under these conditions, leaves were removed for anatomic analysis.

In the *in vitro* treatment, the seeds of crambe were initially washed in running water with neutral detergent. Subsequently, they were transferred into aseptic conditions under laminar flow hood. Next, the seeds were disinfested by immersion in antibiotic solution containing penicillin (10 mg L^{-1}) and rifampicin (10 mg L^{-1}) for 30 minutes (min), followed by 70% alcohol for 1 min, commercially available sodium hypochlorite 50% (active chlorine: 2-2.5%) for 30 min, then washed three times in autoclaved distilled water. The used culture medium was MS½ (Murashige; Skoog, 1962), supplemented with 15 g L^{-1} sucrose and 7 g L^{-1} agar, and the pH was adjusted to 5.8; next, the medium was autoclaved under 1.1 atm and 121 °C, for 20 min. The seeds were inoculated into test tubes of 25x150 mm containing 10 mL of medium, and incubated in growth room with photoperiod of 16/8 hours (light/dark), under fluorescent lamps providing flow of 25.2 µmoL m^{-2} s^{-1} photosynthetic photons, and temperature of 25±1 °C. After 30 days in growth room, leaves were collected for analyses.

The plants used for pre-acclimatization were obtained in a micropropagation process of 120 days (experiment 02). After this period, the first process of pre-acclimatization was to transfer the plants from the test tube into larger closed flasks (80x150 mm) containing culture medium MS¼, with 7.5 g L^{-1} agar, without addition of sucrose, and maintained under the growth room conditions described for the *in vitro* treatment. The evaluation and removal of leaves were carried out after 30 days in these conditions (Acclimatization 01). The second step of the pre-acclimatization was to maintain the plantlets in the closed flasks with autoclaved commercial substrate (Vivato Slim®), nourished with MS solution without sucrose, and kept in a place with light and room temperature. The evaluations and removal of leaves were performed after 30 days in these conditions (Acclimatization 02). After this step, the plants were transferred into plastic beakers (180 ml) with the same soil and greenhouse conditions described for the treatment *in vivo*. The soil was autoclaved and nourished with solution of macro- and micronutrients of the medium MS, being irrigated whenever necessary to maintain the moisture. However, after 10 days, the plants had not survived under these conditions, without possibility of a new evaluation.

Anatomic Analysis

The anatomic analyses were performed in five more developed leaves of each treatment, being analyzed in the midrib region and internervural area. The samples were fixed in formalin-acetic acid-alcohol (FAA_{50}; Johansen, 1940) for 48 hours, stored in ethyl alcohol 70%, later subjected to dehydration in increasing ethyl series (70, 90, 95 and 100%), and embedded in hydroxyethyl methacrylate (Leica®, Germany). The blocks were cross sectioned with help of a rotating microtome, with thickness varying between 8 and 10 µm. The slices were stained with toluidine blue 0.05% in acetate buffer, pH 4.3 (O'brien; Feder; Mccully, 1964), and mounted between slide and coverslip in synthetic resin Entellan®. Measurements of the thickness of the blade, mesophyll, adaxial and abaxial surfaces of the epidermis, palisade parenchyma, spongy parenchyma and midrib region were performed. Also the vascular tissue area of the midrib was measured. The observations and measurements were carried out under microscope Nikon E200 with coupled digital camera, connected to a computer containing the software Tsview® (China). The photo documentation was done in a photomicroscope Nikon 50i (Nikon, Japan). The design for anatomic evaluations was completely randomized, with five repetitions per treatment, each repetition corresponding to one leaf and comprising the mean value of three slides with eight sections each.

Statistical Analysis

All obtained data was subjected to analysis of variance, after verification of normality and homogeneity. After confirming the significance of the treatments, the Tukey test of averages was applied, adopting 1% and 5% probability, with the software Assistat (Silva; Azevedo 2009).

RESULTS AND DISCUSSION

Regarding the general anatomic description of *C. abyssinica*, the leaf is amphistomatic, with uniseriate epidermis constituted by cells of varying shapes and sizes, covered by a thin cuticle. Gland and tector trichomes occur sparsely on both surfaces of the epidermis (data not shown). The mesophyll is dorsiventral, formed by one or two cell layers of palisade parenchyma, and spongy parenchyma with three to four cell layers. Vascular bundles of collateral type and small size occur across the mesophyll. The midrib, in cross section, appears concave-convex, with epidermis formed by round cells. The vascular system in predominantly formed by a single collateral vascular bundle (Figure 1).

The leaf anatomy of *C. abyssinica* is similar to that described for other representatives of Brassicaceae, such as *Brassica napus* L. (colza), *Brassica gravinae* Ten., *Brassica rapa* L. (turnip), *Raphanus sativus* L. (radish), *Diplotaxis tenuifolia* (Ueno, 2011) and *Arabidopsis thaliana* (L.) Heynh (Boeger; Poulson, 2006), with the latter having the difference of presenting mesophyll without differentiation between the palisade and spongy parenchyma.

Comparing the anatomy of the blade in plants cultivated *in vivo* and *in vitro*, as well as during pre-acclimatization, it was verified that the leaves subjected

to acclimatization 01 and 02 presented epidermis showing cells with dehydrated appearance (Figure 1 – C, D, G and H). This fact is probably due to the change in moisture occasioned by the transfer from test tube to larger flasks. According to Seelye, Burge and Morgan (2003), the loss of water by the cells recently transferred to *in vitro* conditions is an early symptom of inadequate adaptation of the plant to these new conditions.

The plantlets cultured *in vitro* demonstrated absence (or reduced deposition) of cuticle on both surfaces in comparison with those cultivated *in vivo* and during pre-acclimatization (Figure 1 – A and B). The absence or reduction of the cuticle layer in leaves of plantlets cultivated under *in vitro* conditions is apparently related to the high moisture inside the test tube (Batagin-Piotto et al., 2012) and the lower light intensity (Calvete et al., 2002). It is important to highlight that the cuticle is extremely important for the pre-acclimatization process of the plantlets, since it helps control the transpiration and the passive loss of water (Taiz; Zeiger, 2006).

The palisade parenchyma in the *in vivo* and *in vitro* treatments was generally constituted by one layer of cells, but at some points two cell layers were present. The cells of this region appeared more elongated under *in vivo* conditions, and more juxtaposed *in vitro* (Figure 1 – A and B), possibly due to the low light intensity that occasioned the investment in a higher number of cells *in vitro.* Abbade et al., (2009), working with *Tabebuia roseo alba* (white ipê – Bignoniaceae), related that the plants *in vivo* presented palisade parenchyma more elongated and juxtaposed than in the *in vitro* conditions. According to Lee et al. (2000), more elongated palisade cells constitute an adaptation of the plant to high light intensity, which explains the leaves *in vivo* presenting higher values than *in vitro* ones.

The spongy parenchyma of the plantlets cultivated *in vivo* exhibited four to five layers of cells and larger intercellular spaces than *in vitro,* which showed from three to four cell layers (Figure 1 – A and B).

In acclimatization (tests 01 and 02), the parenchyma demonstrated loss of cells and disarrangement of its structure in the different substrates (Figure 1 – C, D, G and H). Hydric stress of the plants is generally the biggest problem during the pre-acclimatization process. Though appearing perfect *in vitro*, a plant may present anatomic alterations and deficiencies that hamper the control of transpiration, leading to a fast loss of water (Barboza et al., 2006).

In the midrib of the median region of the leaf blade, the vascular system was normally constituted by a cylindrical vascular bundle, with the possibility of showing more than one of these (Figure 1 – E-H).

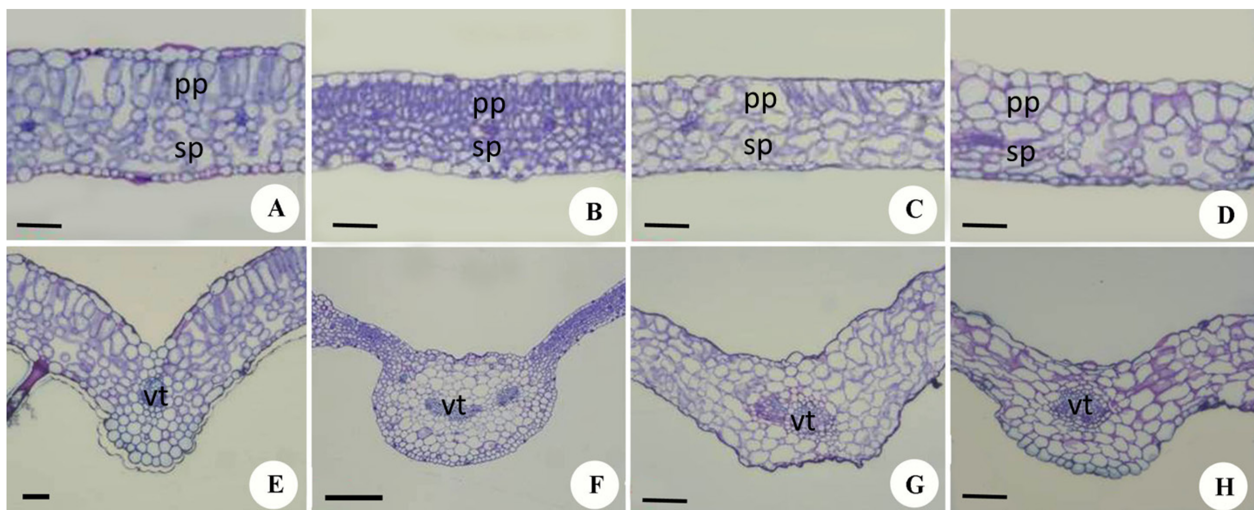

Figure 1 – Cross section of the mesophyll (A-D) and midrib (E-H) of *C. abyssinica* leaves *in vivo* (30 days) (A and E), *in vitro* (30 days) (B and F), and during acclimatization 01 (30 days) (C and G) and acclimatization 02 (60 days) (D and H). pp – palisade parenchyma; sp – spongy parenchyma; vt – vascular tissue. Bar (–) = 100 µm.

According to the percentage of survival before acclimatization (tests 01 and 02), that is, after 120 days of culture *in vitro,* the plantlets of crambe presented 99.1% of survival on average. The plantlets transferred to the treatment 01, after 30 days in these conditions, had a mean survival rate of 93.3%, and in test 02, for further 30 days, showed even better survival rates, reaching 100%. However, after being transferred to transparent plastic beakers and taken to greenhouse, there was no survival after 10 days in these conditions.

Chirinéa et al. (2012), working with plants of *Ficus carica* cv. "Roxo de Valinhos" (common fig – Moraceae), obtained good results with a pre-acclimatization *in vitro,* as also done in this work with crambe. These authors obtained 95% of survival with commercial substrate in greenhouse after realization of a pre-acclimatization for 60 days with medium woody plant medium (WPM), without growth regulators. Pereira and Fortes (2001), following the same pre-acclimatization procedure, obtained positive results in apple tree "M.111" and "Marubakaido" (Rosaceae), with an mean value of 90% in the survival rate for the treatments maintained for 12, 15, 21 and 30 days under *in vitro* conditions.

Table 1 shows the comparison among the anatomic characteristics evaluated in crambe leaves. It is observed that there was no statistical difference as to the thickness of the blade and of the leaf mesophyll, with the highest values being found in plants established under *in vivo* conditions (176.49 and 136.01 µm, respectively). With regards to the thickness in the mesophyll tissues, there was no significant difference in the palisade and in the spongy parenchyma. The thickness of the palisade parenchyma was greater in the leaves cultivated *in vivo* (62.92 µm), and in the spongy parenchyma the highest value was seen in Acclimatization 01 (84.49 µm).

Similar results were reported by Batagin-Piotto et al. (2012), working with *Bactris gasipaes* (Arecaceae). The obtained data also agrees with other studies about acclimatization of micropropagated plants, such as *Fragaria ananassa* Duch. (Rosaceae) (Calvete et al., 2002) and *Quercus suber* L. (Fagaceae) (Romano; Martins-Loucao, 2003), who related smaller differentiation of the mesophyll, thickness of the palisade and spongy parenchyma, besides smaller intercellular spaces, which are modifications frequently observed in leaves of plants *in vitro*, when compared to plants *in vivo*. Romano and Martins-Loucao (2003), also working with eucalypt hybrid (*Eucalyptus grandis* W. Hill ex. Maiden × *E. urophylla* S.T. Blake), found results similar to those of this work, with increase in the differentiation and thickness of tissues occurring with acclimatization.

In the adaxial and abaxial surfaces of the epidermis (Table 1) significant difference occurred as to their thickness. Again, superior values were found in *in vivo* conditions. In the adaxial surface, the value found for this condition (21.05 µm) was statistically superior, followed by the treatment *in vitro* (15.99 µm). In turn, in the abaxial surface, the value for the condition *in vivo* was 16.40 µm, followed once again by the *in vitro* conditions (14.77 µm), without, however, differing statistically.

Table 1 – Mean thickness (µm) of the blade, mesophyll, adaxial and abaxial surfaces of the epidermis, palisade and spongy parenchyma, midrib and area of the vascular bundle (µm^2) of *C. abyssinica* leaves obtained by germination *in vivo* (30 days), *in vitro* (30 days) and during acclimatization 01 (30 days) and 02 (60 days).

Treatments	Blade	Mesophyll	Adaxial surface	Abaxial surface	Palisade parenchyma	Spongy parenchyma	Midrib	Vascular bundle
In vivo	176.49 a	136.01 a	21.05 a	16.40 a	62.92 a	72.56 a	282.27 ab	4490.24 b
In vitro	155.04 a	121.81 a	15.99 b	14.77 a	47.43 a	75.81 a	414.44 a	20066.43 a
Acclim. 01	146.14 a	124.21 a	11.77 c	10.65 b	43.38 a	84.49 a	251.71 b	9387.69 b
Acclim. 02	147.28 a	122.08 a	14.50 bc	11.13 b	50.66 a	76.38 a	267.20 ab	8900.00 b
General mean	156.24	126.03	15.83	13.24	51.10	77.31	303.91	10711.09
CV (%)	13.38	14.76	12.61	13.97	22.60	17.10	29.35	49.52

There is no difference among means followed by the same letter, in the column, by Tukey test at 5%.

Regarding the midrib, its thickness was greater under *in vitro* conditions (414.44 µm), without differing significantly from the *in vivo* conditions (282.27 µm) and acclimatization 02 (267.20 µm) (Table 1). According to Alves and Angyalossy-Alfonso (2000), environmental factors affect the dimensions and even the arrangement of the vascular elements, in an attempt to guarantee an increase in the security of transport when the plant is subjected to some type of stress.

The area of the vascular bundle was superior and significantly different under *in vitro* conditions (20066.43 μm^2) (Table 1). It should be highlighted that, although no statistical difference was observed, the values obtained in the acclimatized material (tests 01 and 02) are much higher in relation to those of *in vivo* conditions, which demonstrates an attempt to overcome some type of stress, be it hydric or nutritional. Another important factor is that, in the *in vivo* treatment, the presence of more than one vascular bundle in the midrib was not so frequent as *in vitro* and during pre-acclimatization (Figure 1E and 1F), which also explains superior values under these conditions. Contrary to what was found in crambe, the vascular bundles of plants cultivated *in vitro* and acclimatized were more reduced than those of plants *in vivo* in leaves of *Rollinia mucosa* (Annonaceae), in which the vascular system presented a reduced number of conducting elements (Albarello et al., 2001). The reduction in vascular tissue, especially the xylem, is a typical characteristic of plants of humid environments (Mayer et al., 2008), as is the case of *in vitro* conditions. It was verified that the leaves of crambe had their highest values with regards to area of the vascular bundle in *in vitro* conditions and pre-acclimatization.

Various histologic studies have demonstrated that the vegetative organs of plants developed *in vitro* present tissues and structures which are little differentiated when compared to plants cultivated in greenhouse (Apóstolo; Brutti; Llorente, 2005; Louro et al., 2003). In the leaf anatomy of crambe plants cultivated *in vivo* and *in vitro,* little difference was observed between their structures, although the number of cells in the mesophyll is higher in the *in vitro* environment.

CONCLUSION

Knowledge about the morphological alterations in plants developed *in vitro* is fundamental for the survival of plants originated in controlled environments to under natural conditions, and thereby contribute to establishing efficacious micropropagation protocols. Consequently, the alterations observed in the structure and thickness of the leaves from the acclimatization treatments 01 and 02 hampered the process of seedling establishment *ex vitro.*

REFERENCES

ABBADE, L. C. et al. Anatomia foliar de ipê-branco branco (*Tabebuia roseo alba* (Ridl.) Sand.) – Bignoniaceae, proveniente do cultivo *ex vitro* e *in vitro*. **Acta Scientiarum. Biological Sciences**, Maringá, 31(3):307-311, 2009.

ALBARELLO, N. et al. Anatomia foliar de *Rollinia mucosa* Jacq. Baill. (Annonaceae) sob condições de cultivo *in vivo* e *in vitro*. **Revista Brasileira de Plantas Medicinais**, 4(1):35-46, 2001.

ALVES, E. S.; ANGYALOSSY-ALFONSO, V. Ecological trends in the wood of some Brazilian species: Growth rings and vessels. **International Association of Wood Anatomists Journal**, 21(1):3-30, 2000.

APÓSTOLO, N. M.; BRUTTI, C. B.; LLORENTE, B. E. Leaf anatomy of *Cynara scolymus* L. in successive micropropagation stages. **In Vitro Cellular & Developmental Biology – Plant**, 41(1):307-313, 2005.

APPEZZATO-DA-GLÓRIA, B.; VIEIRA, M. L. C.; DORNELAS, M. C. Anatomical studies of *in vitro* organogenesis induced in leaf-derived explants of passionfruit. **Pesquisa Agropecuária Brasileira**, 34(11):2007-2013, 1999.

BARBOZA, S. B. S. C. et al. Anatomia foliar de plantas micropropagadas de abacaxizeiro. **Pesquisa Agropecuária Brasileira**, 41(2):185-194, 2006.

BATAGIN-PIOTTO, K. D. et al. Anatomical analysis of peach palm (*Bactris gasipaes*) leaves cultivated *in vitro*, *ex vitro* and *in vivo*. **Brazilian Journal of Botany**, 35(1):71-78, 2012.

BOEGER, M. R. T.; POULSON, M. E. Efeitos da radiação ultravioleta-B sobre a morfologia foliar de *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). **Acta Botanica Brasilica**, 20(2):329-338, 2006.

BRAGA, F. T. et al. Alterações morfofisiolócias de plantas de abacaxizeiro influenciadas por diferentes substratos durante o processo de aclimatização. **Ciência e Agrotecnologia**, 35(5):863-868, 2011.

CALVETE, E. O. et al. Análises anatômicas e da biomassa em plantas de morangueiro cultivadas *in vitro* e *ex vitro*. **Horticultura Brasileira**, 20(4):649-653, 2002.

CAPELLE, A.; TITTONEL, E. D. Crambe, a potential non-food oil crop: Production. **Agro Food Industry Hi-Tech**, 10(1):22-27, 1999.

CHANDRA, S. et al. Acclimatization of tissue cultured plantlets: From laboratory to land. **Biotechnology Letters**, 32(1):1199-1205, 2010.

CHHIKARA, S. et al. Development of an *Agrobacterium*-mediated stable transformation method for industrial oilseed crop *Crambe abyssinica* 'BelAnn'. **Industrial Crops and Products**, 37(1):457-465, 2012.

CHIRINÉA, C. F. et al. Acclimatization and leaf anatomy of micropropagated fig plantlets. **Revista Brasileira de Fruticultura**, 34(4):1180-1188, 2012.

DOUSSEAU, S. et al. Anatomia foliar de *Tabebuia serratifolia* (Vahl.) NIch. (Bignoniaceae) propagadas *in vitro*, *in vivo* e durante a aclimatização. **Ciência e Agrotecnologia**, 32(6):1694-1700, 2008.

FERMINO JUNIOR, P. C. P.; SCHERWINSKI-PEREIRA, J. E. Características anatômicas de folhas de Teca (*Tectona grandis* L.) desenvolvidas sob condições de cultivo *in vitro* e *ex vitro.* **Evidência**, 9(1-2):17-28, 2009.

GAO, H. B. et al. Studies on the Plant regeneration from single cell culture of *Crambe abyssinica*. **Hereditas**, 20(1):50-52, 1998.

HAZARIKA, B. N. Morpho-physiological disorders in *in vitro* culture of plants. **Scientia Horticulturae**, 108(1):105-120, 2006.

HRONKOVA, M. et al. The role of abscisic acid in acclimation of plants cultivated *in vitro* to *ex vitro* conditions. **Biologia Plantarum**, 46(1): 535-541, 2003.

JOHANSEN, D. A. **Plant microtechnique**. 2. ed. New York: Mc-Graw-Hill, 1940. 523p.

KADLECEK, P. et al. Importance of *in vitro* pretreatment for *ex vitro* acclimatization and growth. **Plant Science**, 161(1):695-701, 2001.

KOZAI, T. Micropropagation under photoautotrophic conditions. In: DEBERGH, P. C.; ZIMMERMAN, R. H. (eds.). **Micropropagation: Technology and Application**, Kluwer, Dordrecht, 1991. p.447-469.

LEE, D. W. et al. Effects of irradiance and spectral quality on leaf structure and function in seedlings of two Southeast Asian Hopea (dipterocarpaceae) species. **American Journal of Botany**, 87(4):447-455, 2000.

LI, X. et al. Highly efficient *in vitro* regeneration of the industrial oilseed crop *Crambe abyssinica*. **Industrial Crops and Products**, 33(1):170-175, 2011.

LI, X. et al. Genetic transformation of the oilseed crop *Crambe abyssinica*. **Plant Cell Tissue and Organ Culture**, 100(1):149-156, 2010.

LOURO, R. P. et al. Ultrastructure of *Eucalyptus grandis* x *E.urophylla* plants cultivated *ex vitro* in greenhouse and field conditions. **Trees**, 17(1):11-22, 2003.

MATHUR, A. et al. Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum*. **African Journal of Biotechnology**, 7(1):1046-1053, 2008.

MAYER, J. L. S. et al. Anatomia comparada das folhas e raízes de *Cymbidium* Hort. (Orchidaceae) cultivadas *ex vitro* e *in vitro*. **Acta Botanica Brasilica**, 22(2):323-332, 2008.

MURASHIGE, T.; SKOOG, F. A. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, 15(3):473-497, 1962.

MURPHY, D. J. Production of novel oils in plants. **Current Opinion in Biotechnology**, 10(1):175-180, 1999.

O'BRIEN, T. P.; FEDER, N.; MCCULLY, M. E. Polychromatic staining of plant cell walls by toluidine blue. **Protoplasma**, 59(2):368-373, 1964.

PALMER, C. D.; KELLER, W. A. Somatic embryogenesis in *Crambe abyssinica* Hochst. Ex R.E. Fries using seedling explants. **Plant Cell Tissue and Organ Culture**, 104(1):91-100, 2011.

PEREIRA, J. E. S.; FORTES, G. R. L. Multiplicação e aclimatização da macieira influenciada pelo tipo de explante e pelo tempo de permanência em meio de cultura de enraizamento. **Revista Brasileira Fruticultura**, 23(1):417-420, 2001.

PREECE, J. E.; SUTTER, E. G. Acclimatization of micropropagated plants to the greenhouse and field. In: DEBERGH, P. C.; ZIMMERMAN, R. H. (eds.). **Micropropagation: Technology and Application**, Kluwer, Dordrecht, 1991. p. 71-93.

ROMANO, A.; MARTINS-LOUCAO, M. A. Water loss and morphological modifications in leaves during acclimatization of cork oak micropropagated plantlets. **Acta Horticulturae**, 616(1):439-442, 2003.

SEELYE, J. F.; BURGE, G. K.; MORGAN, E. R. Acclimatizing tissue culture plants: Reducing the shock. **Combined Proceedings International Plant Propagators' Society**, 53(1): 85-90, 2003.

SILVA, F. de A. S. E.; AZEVEDO, C. A. V. de. **Principal Components Analysis in the Software Assistat-Statistical Attendance**. In: WORLD CONGRESS ON COMPUTERS IN AGRICULTURE, 7, Reno-NV-USA: American Society of Agricultural and Biological Engineers, 2009.

TAIZ, L.; ZEIGER, E. **Fisiologia vegetal**. 3ed. Porto Alegre, Artmed. 2006.722p.

UENO, O. Structural and biochemical characterization of the C3–C4 intermediate *Brassica gravinae* and relatives, with particular reference to cellular distribution of Rubisco. **Journal of Experimental Botany**, 62(15):5347-5355, 2011.

VARGAS-LOPEZ, J. M. et al. Processing of Crambe oil and isolation of erucic acid. **Journal of the American Oil Chemists' Society**, 76(1):801-809, 1999.

WANG, Y.; PENG, P. Intergeneric hybridization between Brassica species and *Crambe abyssinica*. **Euphytica**, 101(1):1-7, 1998.

WANG, Y. P. et al. Cytogenetic characterization and fae1 gene variation in progenies from asymmetric somatic hybrids between *Brassica napus* and *Crambe abyssinica*. **Genome**, 47(1):724-731, 2004.

WANG, Y. P.; SONNTAG, K.; RUDLOFF, E. Development of rapeseed with high erucic acid content by asymmetric somatic hybridization between *Brassica napus* and *Crambe abyssinica*. **Theoretical and Applied Genetics**, 106(1):1147-1155, 2003.

ZOBAYED, S. M. A.; ARMSTRONG, J.; ARMSTRONG, W. Leaf anatomy of *in vitro* tobacco and cauliflower plantlets as affected by different types of ventilation. **Plant Science**, 161(1):537-538, 2001.