27. Sabani, W., Rahmadewi, D. P., Rahmi, K. I. N., Priyatna, M. and Kurniawan, E., Utilization of MODIS data to analyze the forest/land fires frequency and distribution (case study : Central Kalimantan Province). *IOP Conf. Ser.: Earth Environ. Sci.*, 2019, **243**, 012032.
28. Tanpipat, V., Honda, K. and Nuchaiya, P., MODIS hotspot validation over Thailand. *Remote Sensing*, 2009, **1**, 1043–1054.
29. Huete, A. R., Liu, H. Q., Batchily, K. and van Leeuwen, W., A comparison of vegetation indices over a global set of TM images for EOS-MODIS. *Remote Sensing Environ.*, 1997, **59**, 440–451.
30. Huffman, G. J., Bolvin, D. T., Braithwaite, D., Hsu, K., Joyce, R., Xie, P. and Yoo, S. H., NASA global precipitation measurement (GPM) integrated multi-satellite retrievals for GPM (IMERG). Algorithm Theoretical Basis Document (ATBD) Version, 2015, 4, p. 26.
31. Hastie, T. and Tibshirani, R., Generalized additive models. *Stat. Sci.*, 1986, **1**, 297–318.
32. Mahato, S., Pal, S. and Ghosh, K. G., Effect of lockdown amid COVID-19 pandemic on air quality of the megacity Delhi, India. *Sci. Total Environ.*, 2020, **730**, 139086.
33. Sharma, S., Zhang, M., Anshika, Gao, J., Zhang, H. and Kota, S. H., Effect of restricted emissions during COVID-19 on air quality in India. *Sci. Total Environ.*, 2020, **728**, 138878.
34. Dantas, G., Siciliano, B., França, B. B., da Silva, C. M. and Arbilla, G., The impact of COVID-19 partial lockdown on the air quality of the city of Rio de Janeiro, Brazil. *Sci. Total Environ.*, 2020, **729**, 139085.
35. Kerimray, A. *et al.*, Assessing air quality changes in large cities during COVID-19 lockdowns: the impacts of traffic-free urban conditions in Almaty, Kazakhstan. *Sci. Total Environ.*, 2020, **730**, 139179.
36. Nakada, L. Y. K. and Urban, R. C., COVID-19 pandemic: impacts on the air quality during the partial lockdown in São Paulo state, Brazil. *Sci. Total Environ.*, 2020, **730**, 139087.
37. Nemani, R. R., Pierce, L. E. and Running, S. W., Developing satellite-derived estimates of surface moisture status. *J. Appl. Meteor.*, 1993, **32**(3), 548–557.
38. Li, L., Zhang, L., Xia, J., Gippel, C. J., Wang, R. and Zeng, S., Implications of modelled climate and land cover changes on runoff in the middle route of the south to north water transfer project in China. *Water Resour. Manage.*, 2015, **29**, 2563–2579.
39. Mildrexler, D. J., Zhao, M., Cohen, W. B., Running, S. W., Song, X. P. and Jones, M. O., Thermal anomalies detect critical global land surface changes. *J. Appl. Meteorol. Climatol.*, 2018, **57**, 391–411.
40. Vlassova, L., Pérez-Cabello, F., Mimbrero, M. R., Llovería, R. M. and García-Martín, A., Analysis of the relationship between land surface temperature and wildfire severity in a series of Landsat images. *Remote Sensing*, 2014, **6**, 6136–6162.
41. Giglio, L., Characterization of the tropical diurnal fire cycle using VIRS and MODIS observations. *Remote Sensing Environ.*, 2007, **108**(4), 407–421.

ACKNOWLEDGEMENTS. We thank the Chairman, Indian Space Research Organisation, Department of Space, Government of India for support. We also thank FIRMS for active fire points, LP DAAC for spatial data of NDVI, EVI and LST and GES DISC for precipitation and soil moisture data.

Received 13 May 2020; revised accepted 19 May 2020

doi: 10.18520/cs/v119/i2/390-398

Foliar micromorphometric adaptations of micropropagated plants of *Oldenlandia herbacea* (L.) Roxb. – an important medicinal herb

J. Revathi[1], M. Manokari[2], S. Priyadharshini[1] and Mahipal S. Shekhawat[1,*]

[1]Biotechnology Laboratory, Kanchi Mamunivar Government Institute for Postgraduate Studies and Research, Puducherry 605 008, India
[2]Siddha Clinical Research Unit, Central Council for Research in Siddha, Palayamkottai, Tirunelveli 727 002, India

An effective *in vitro* regeneration protocol is essential to improve the natural population of conservation-prioritized plants species. The micropropagation techniques are considered cost-effective if the survival chance of tissue-cultured plants is excellent in field conditions. Comparative foliar micromorphometric characteristics were analysed in this study, to determine the sequential developmental adaptations of foliages of *Oldenlandia herbacea* plantlets under *in vitro* and field conditions. The leaf constants showed considerable variations in stomatal morphology, type and density (decreased from 60.0 to 40.75), vein islet density (increased from 8.3 to 13.5) and raphides density (increased from 20.9 to 36.0) in the foliage of tissue-cultured and field-transferred plantlets. The micromorphometric changes reflect the developmental improvements taking place in the greenhouse and field transplantation of *O. herbacea* plants, which are essential for the survival of plantlets under natural conditions.

Keywords: Foliar micromorphology, *in vitro* regeneration, medicinal herb, micropropagation, *Oldenlandia herbacea*.

OLDENLANDIA herbacea (L.) Roxb. (family Rubiaceae), commonly known as chayaparpatika, is considered as the most important medicinal plant for its febrifuge, anthelmintic, expectorant, stomachic and anti-inflammatory properties[1]. It is a seasonal plant which completes its life cycle in 3–4 months. Conventionally, this plant is propagated only by seeds. The plants are being uprooted by the traditional drug practitioners before seed-setting; therefore, the population of *Oldenlandia* species has depleted sharply in recent years[2].

In vitro regeneration techniques offer valuable prospects in large-scale production of medicinal plants using bare minimum starting materials from the donor plant[3]. This also reduces the impact of over-exploitation on the native population of medicinal plants. However, the extensive use of *in vitro* technology is constrained due to difficulties in the survivorship of micropropagated plantlets under natural conditions after transplantation[4].

*For correspondence. (e-mail: smahipal3@gmail.com)

The anatomical and physiological disorders induced by laboratory conditions hinder the growth and survival rate of plantlets in greenhouse and field conditions after transplantation[5]. The heterotrophic conditions of the *in vitro* environment enriched with sugar in the culture medium are known to induce morphological and physiological changes in laboratory-grown plants[6]. The external supply of carbohydrates is responsible for reduced photosynthetic ability of *in vitro*-grown plants, which leads to reduction in survival rate during acclimatization[7].

The leaf characteristics like stomatal type and density, veins and vein-islets density, trichomes, raphides, crystals, etc. are directly responsible for the effective physiological and anatomical developments taking place, while the *in vitro* regenerated plants are transferred to field environments. The variations in the intensity of light, temperature, relative humidity and carbon dioxide concentration from *in vitro* to *in vivo* environments affect the normal developmental processes of plants[8]. The ambient *in vitro* environments induce certain morpho-developmental abnormalities in the foliar apparatus, which is involved in effective stomatal regulation and photosynthetic efficiency of micropropagated plants[9]. These abnormalities must be corrected in the greenhouse before transplantation of *in vitro*-propagated plantlets to natural habitats.

The micromorphological and morphometric characteristics of leaves are direct indications of environmental changes; therefore, the foliar micromorphometric analysis of micropropagated plants at subsequent stages may help understand the acclimation process[4]. This knowledge could further assist in proper handling of micropropagated plantlets during field trials under stressful natural conditions.

This study is a comparative micromorphometric analysis of leaves of tissue-cultured and naturally acclimatized plantlets of *O. herbacea*, to assess the range of similarities and variations related to ecological adaptation of regenerated plantlets. This could help increase the survival chances of tissue culture-raised plants during field transfer.

Phenotypically healthy *O. herbacea* plants were collected from undisturbed wild areas of the east coast of Puducherry (11.9416°N lat. and 79.8083°E long., altitude 15 m amsl), India. *In vitro* shoots of *O. herbacea* were generated using nodal segments as explants. MS basal medium[10] supplemented by 6-benzylaminopurine (BAP) was found optimum for induction of axillary buds and shoot proliferation from nodes. The rate of shoot proliferation was accelerated by formulating MS medium with BAP, kinetin (6-furfurylaminopurine) and indole-3 acetic acid (IAA). Rhizogenesis of shoots was performed by *in vitro* and *ex vitro* methods using indole-3 butyric acid (IBA) and maintained in the greenhouse for four weeks. Acclimatization was achieved in the greenhouse and plantlets were successfully transferred to the field.

Foliar micromorphometric analysis was done at *in vitro* and field environments. Leaf constants such as stomatal pattern, frequency, density, index and type of stomata, and leaf architectural parameters, including venation pattern and its density, density and type of trichomes, crystals and raphides were studied. Leaves randomly selected from multiplication stage under laboratory conditions and from the field after six weeks of transplantation were subjected to micromorphometric scrutiny[11]. The foliages from both environments were carefully collected from third to seventh leaves to compare the developmental adaptation to predict the possibility of survivability. Paradermal sections were obtained manually using the standard method[12] from the entire foliar apparatus to evaluate the developmental changes in leaf constants. Foliar architectural developments were studied by fixing the fresh leaves in formalin : acetic acid : ethanol fixative in the ratio of 1 : 1 : 3 (v/v). The chlorophyll content was removed by maintaining the leaves in 70% ethanol (v/v) for 12–24 h and followed by 5% (w/v) NaOH for 24–48 h. The treated leaves were washed thoroughly and used to study architectural parameters such as characteristics of veins, trichomes, crystals and raphides. Finally, the leaf materials were stained with safranin for 5 min. Light-microscopic observations were used to describe the developmental adaptations of the leaves.

The significance of variance in each experiment was tested by one-way ANOVA. Data comparison was done by Duncan's Multiple Range Test at $P < 0.05$ using SPSS software, version 16.0. The findings were expressed as mean ± standard error and the foliar micromorphometric parameters were calculated in micrometers (μm).

The foliar micromorphological studies of leaf constants such as stomatal type, frequency, stomatal density and its index, vein architectural pattern, vein density, trichomes,

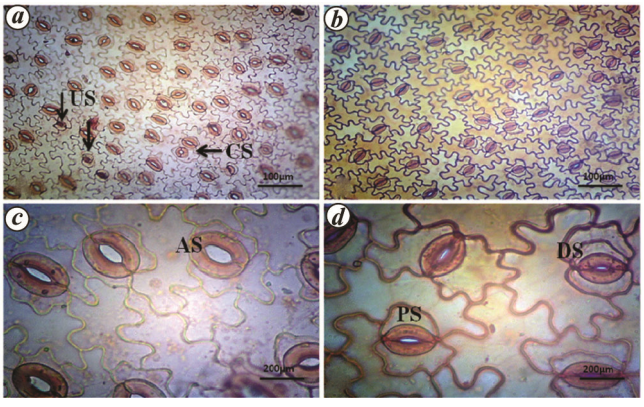

Figure 1. ***a***, Frequency and density of stomatal apparatus on abaxial surface of leaves under *in vitro* conditions (US, underdeveloped stomata, CS, contiguous stomata). ***b***, Frequency, orientation and density of stomatal apparatus on abaxial surface of leaves under field conditions. ***c***, ***d***, Enlarged view of stomatal apparatus under laboratory and field conditions showing anisocytic stomata (AS), paracytic stomata (PS) and paracytic with dicyclic subsidiary on one side (DS). (Scale bar = 100 and 200 μm).

Table 1. Micromorphometric developments in the foliages of micropropagated *Oldenlandia herbacea* plants from *in vitro* to field conditions

Quantitative micromorphometry (per mm^2)	Under laboratory conditions (mean ± SE)	Under field conditions (mean ± SE)
Stomatal density	60.0 ± 0.38	40.75 ± 0.20
Stomatal index	30.76 ± 0.32	25.0 ± 0.29
Number of vein-islets	8.3 ± 0.25	13.5 ± 0.33
Number of veinlets terminations	7.99 ± 0.18	11.65 ± 0.14
Raphide density	20.9 ± 0.19	36.0 ± 0.27

Note: Values in each category are the quantitative mean ± standard error of 60 experiments.

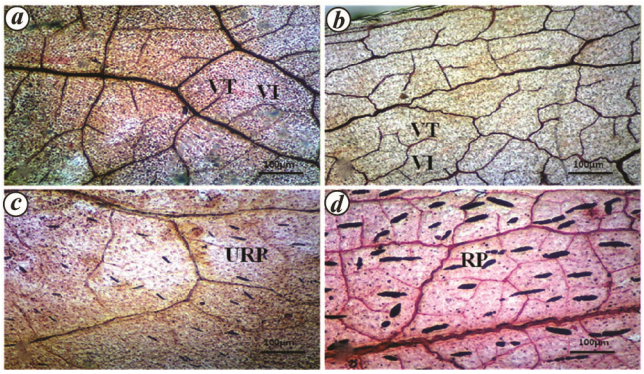

Figure 2. ***a***, Photomicrograph representing poorly developed vein architecture in *in vitro* regenerated leaves under laboratory conditions. ***b***, Enhanced leaf-vein density after field transplantation (VI, Vein-islet, VT, veinlet termination) (scale bar = 100 µm). ***c***, Structure and density of raphides observed under *in vitro* environment (URP, underdeveloped raphides). ***d***, Development of raphides (RP) under field conditions.

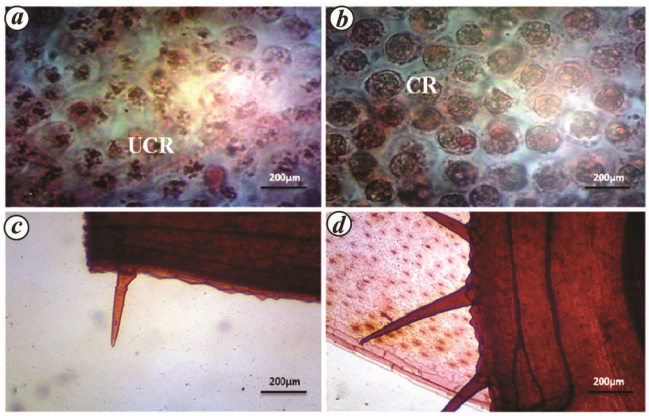

Figure 3. ***a***, Calcium oxalate crystals observed in leaves at the multiplication stage (UCR, underdeveloped crystals). ***b***, Structurally ideal calcium oxalate crystals (CR) observed after field transplantation (scale bar = 200 µm). ***c***, Simple and underdeveloped trichome with *in vitro* raised leaf. ***d***, Well-developed multiple trichomes on the leaf surface of field-transferred plantlets.

calcium oxalate crystals and raphide densities of *O. herbacea* under *in vitro* and field environment could contribute significantly to understand the changes taking place in the leaves when the tissue culture-raised plantlets are shifted to natural habitats.

The light-microscopic studies of paradermal sections of leaves developed in the field environment showed the presence of compact, amoeboidal-shaped epidermal cells with wavy walls, whereas the epidermal cells of tissue-cultured leaves were less wavy and unorganized. The leaves were hypostomatic with squarish and rectangular adaxial epidermal cells. *In vitro* leaves possessed irregularly oriented stomata facing all the directions, but well-developed stomata with regular distribution and facing almost the same direction were observed in the field-transferred plants (Figure 1 *a* and *b*). Three types of stomata, namely paracytic, paracytic with dicyclic subsidiary on one side and anisocytic stomata were recognized in this species pertaining to the arrangement of the subsidiary cells. Paracytic and anomocytic types of stomata were prevalent with the tissue-cultured foliages, but paracytic stomata were observed predominantly in field-established plants (Figure 1 *c* and *d*). Stomatal abnormalities like contiguous stomata and disorganized guard cells were detected under laboratory conditions (Figure 1 *a*), which were completely absent in the foliages of acclimatized plantlets under natural conditions. The stomatal density and stomatal index under *in vitro* environment were recorded as 60.0 and 30.76, which gradually decreased to 40.75 and 25.0 respectively, in field environment (Table 1). The presence of paracytic type of stomata in *O. herbacea* has also been reported by Bahadur *et al.*[13]. The stomatal density and their functions are directly influenced by external factors like intensity of light, concentration of CO_2, etc.[14].

Reticulate venation was observed in the foliage of *O. herbacea*. The vein-islets were open, polygonal-shaped and less dense (8.3) in the *in vitro*-grown leaves, but these were distinct and rhomboidal with higher density (13.5) in field-grown plants. The number of veinlet terminations was fewer (7.99) and underdeveloped for *in vitro* leaves, but branched, fully developed and higher veinlet terminations (11.65) were exhibited in field-transferred plants (Figure 2 *a* and *b* and Table 1). This indicates the development of distinct venation pattern during the hardening process by plants, which helps them survive under harsh environmental conditions. The formation of veinlet terminations and closed venation pattern enhance the rate of photosynthesis and transport of minerals to the entire foliar apparatus, which help in acclimation of plantlets[2].

The raphides and crystals in the foliages were made up of calcium oxalate (genus-specific). This feature of *O. herbacea* is common with other members of the family

Rubiaceae. The raphides in *in vitro*-regenerated leaves were small, underdeveloped and less frequent (20.9), but were well organized and increased in numbers (36.0) in the field (Figure 2 *c* and *d*, and Table 1). The crystal sand particles were large in size, more in number and distributed equally in the leaves under field environment, but lesser number of sand crystals was observed in the *in vitro*-grown leaves (Figure 3 *a* and *b*). Calcium oxalate crystals were predominantly distributed in axial parenchyma. The biological activities of calcium oxalate crystals in plants have been discussed in several studies[15]. The functions of these structures are related to calcium regulation and defence mechanism of the plants[16].

The leaves of *O. herbacea* were glabrous with serrate papillate margins. Unicellular and uniseriate hairs were observed on the major veins (midrib) of abaxial surface of the leaves. These were totally absent on the secondary veins. Trichomes were well developed and more on the surface of the foliages developed after transplantation to natural conditions, but few underdeveloped trichomes were detected in the *in vitro*-raised leaves (Figure 3 *c* and *d*). The presence of hairs on the leaves provides a stable microclimate to reduce water loss from the leaf surface due to excessive solar radiation[17]. Therefore, the occurrence of well-developed trichomes on major veins may help control water loss directly from the vascular tissues of the leaves, and this may help in acclimatization of the plantlets.

The microstructural developments with the foliages of tissue-cultured and nature-acclimatized plants of *O. herbacea* have been described here. After acclimatization, the leaves developed abundant hairs, highly organized stomata and dense raphides, which confirmed the foliar micromorphological adaptation of plantlets of *O. herbacea* towards natural conditions. Thus, this study could help in estimating the survival percentage of micropropagated plantlets under natural environments.

Conflict of interest. The authors declare that they have no conflict of interest.

1. Rao, G. S., Nagaraju, G., Mande, A., Kumar, K. S. and Yesubabu, B., *In vivo* anti-inflammatory activity of ethanolic extract of *Oldenlandia herbacea*. *Asian. J. Phytomed. Clin. Res*., 2013, **1**, 14–19.
2. Revathi, J., Manokari, M. and Shekhawat, M. S., Optimization of factors affecting *in vitro* regeneration, flowering, *ex vitro* rooting and foliar micromorphological studies of *Oldenlandia corymbosa* L.: a multipotent herb. *Plant Cell Tiss. Org. Cult*., 2018; http://dx.doi.org/10.1007/s11240-018-1395-8.
3. Kashyap, A., Penak, S. M., Saha, A. and Singh, B. R., *In vitro* plant development of *Eleusine coracana* via indirect organogenesis and somatic embryogenesis using mature seeds as explants. *Curr. Sci*., 2018, **115**(1), 91–98.
4. Chandra, S., Bandopadhyay, R., Kumar, V. and Chandra, R., Acclimatization of tissue cultured plants: from laboratory to land. *Biotechnol. Lett*., 2010, **32**, 1199–1205.
5. Iarema, L., Cruz, A. C. F., Saldanha, C. W., Dias, L. L. C., Vieira, R. F., Oliveira, E. J. and Otoni, W. C., Photoautotrophic propagation of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. *Plant Cell Tiss. Org. Cult*., 2012, **110**, 227–238.
6. Xiao, Y., Niu, G. and Kozai, T., Development and application of photoautotrophic micropropagation plant system. *Plant Cell Tiss. Org. Cult*., 2011, **105**, 149–158.
7. Shin, K. S., Park, S. Y. and Paek, K. Y., Sugar metabolism, photosynthesis, and growth of *in vitro* plantlets of *Doritaenopsis* under controlled microenvironmental conditions. *In vitro Cell. Dev. Biol.–Plant*, 2013, **49**, 445–454.
8. Pospisilova, J., Synkova, H., Haisel, D. and Semoradova, S., Acclimation of plantlets to *ex vitro* conditions: effects of air humidity, irradiance, CO_2 concentration and abscisic acid (a review). *Acta Hortic*., 2007, **748**, 29–38.
9. da Silva, A. B., da Silva, J. M. S. F., da Silva, J. A. S. and Togoro, A. H., Foliar anatomy and *in vitro* growth of *Cattleya* at different concentrations of Kefir, Knudson medium and sucrose. *Biosci. J*., 2011, **27**, 896–901.
10. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*., 1962, **15**, 473-497.
11. Shekhawat, M. S., Kannan, N. and Manokari, M., *In vitro* propagation of traditional medicinal and dye yielding plant *Morinda coreia* Buch.-Ham. *S. Afr. J. Bot*., 2015, **100**, 43–50.
12. Johansen, D. A., *Plant Microtechniques*, McGraw Hill, New York, USA, 1940, 1st edn.
13. Bahadur, B., Rajagopal, T. and Ramayya, N., Studies on the structural and developmental variation and distribution of stomata in the Rubiaceae. *Bot. J. Linn. Soc*., 1971, **64**, 295–310.
14. Casson, S. and Gray, J. E., Influence of environmental factors on stomatal development. *New Phytol*., 2008, **178**, 9–23.
15. Ilarslan, H., Palmer, R. G. and Horner, H. T., Calcium oxalate crystals in developing seeds of soybean. *Ann. Bot*., 2001, **88**, 243–257.
16. Korth, K. L. *et al.*, *Medicago truncatula* mutants demonstrate the role of plant calcium oxalate crystals as an effective defense against chewing insects. *Plant Physiol*., 2006, **141**, 188–195.
17. Riederer, M. and Schreiber, L., Protecting against water loss: analysis of the barrier properties of plant cuticles. *J. Exp. Bot*., 2001, **52**, 2023–2032.

ACKNOWLEDGEMENT. We thank the Department of Science, Technology and Environment, Government of Puducherry for providing financial support (No. 10/DSTE/GIA/RP/JSA-I/2013/213).

Received 20 July 2018; revised accepted 29 January 2020

doi: 10.18520/cs/v119/i2/398-401