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BRIEF COMMUNICATION

Stomatal characteristics during micropropagation of *Wrightia tomentosa*

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Abstract

A deviation from usually found characteristics of stomata in *Wrightia tomentosa* was noted during *in vitro* propagation. Increase in stomatal frequency in leaves of plants grown *in vitro* was observed with 29.4 % malformed stomata. The stomata were spherical, wide open, did not close in detached leaves even after 3 h. The leaves exhibited 93.4 % total water loss during 3-h period. Stomatal frequency, percentage of malformed stomata and rate of water loss declined in subsequent rooting phase. Nevertheless, for high survival rate plantlets were hardened under gradually decreasing air humidity either in partially opened glass bottles containing *Soilrite*™ moistened with ¼ Murashige and Skoog nutrients or in pots covered with polyethylene bags. The stomatal characteristics of hardened plants were comparable to seedlings. Survival rate was more than 95 %.

Additional key words: *ex vitro* transfer, hardening, *in vitro* culture, stomatal frequency, water relations.

The transfer of woody plant species from *in vitro* vessels to *ex vitro* conditions is considered to be a critical stage in successful micropropagation. The major reason for high mortality rate during *ex vitro* transfer lies in excessive water loss attributed to poor stomatal functioning (Blanke and Belcher 1989), reduced leaf epicuticular wax (Sutter and Langhans 1979) and high stomatal densities (Desjardins *et al*. 1988). Hronková *et al*. (2003) have reported that the acclimation of *in vitro* derived plants to *ex vitro* conditions affected the stomata on adaxial and abaxial sides differently. Failure of stomata to close in response to darkness, applied ABA, or high CO_2 concentrations has also been reported in apple and cauliflower plants propagated *in vitro* (Brainerd and Fuchigami 1982). The cause of failure of stomata to close could be due to guard cell wall properties (Ziv *et al*. 1987), the deformation of stomata (Blanke and Belcher 1989) or the K^+ transport across the guard cells (Assmann 1993). Manipulation of irradiance and relative humidity (RH) in *Rosa multiflora* induced changes in stomatal and epidermal cell anatomy (Capellades *et al*. 1990).

Micropropagation of *Wrightia tomentosa*, a threatened tree species of Aravallis in Rajasthan, India has been reported (Purohit *et al*., 1996). Despite high production rate, *in vitro* plants exhibited severe water loss and thus very high mortality during weaning period. Present study investigates stomatal features, relative water loss during different phases of micropropagation and methods for successful hardening of the plantlets for their large-scale production.

Shoot cultures were initiated from aseptically grown seedlings of *W. tomentosa* according to the method described by Purohit *et al* (1996). Shoots induced from cotyledonary nodes on Murashige and Skoog (1962; MS) medium containing 22.2 μM 6-benzylaminopurine (BAP) were repeatedly subcultured every 3 weeks on lower concentrations of BAP (8.9 μM) in the medium. Elongated shoots (2 - 3 cm) were rooted *in vitro* by giving pulse treatment of pre-autoclaved indolebutyric acid (IBA) solution (22.2 μM) for 10 min to cut ends followed by their implantation on ¼ MS salt medium. All the cultures were kept in controlled conditions of temperature (28 ± 2 °C), irradiance (45 μmol m^{-2} s^{-1} for 16-h photoperiod) and 60 - 70 % RH. Although more than 10 000 plantlets were produced during micro-propagation but in the present study 50 plants each were subjected to the following three different modes of hardening: *1*) rooted plants were directly transferred to

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Abbreviations: BAP - 6-benzylaminopurine; DPX - dibutylpthalate xylol; IBA - indolebutyric acid; MS - Murashige and Skoog; RH - relative humidity.
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open pots; *2*) the plants in pots were covered with polyethylene bags for 15 d, followed by gradual opening and complete removal after next 15 d; 3) rooted plants were hardened *in vitro* in culture bottles containing autoclaved *Soilrite*™ moistened with ¼ MS nutrients. After 30 d, caps were loosened, then partially opened and finally removed in the next 30 d. Plantlets were allowed to remain in open bottles for next 15 d. All these operations were carried out in hardening unit under natural daylight (approx. 70 μmol m^{-2} s^{-1}) where temperature of 28 ± 2 °C and RH decreasing from 80 to 40 % were maintained using Fan-Pad evaporative cooling system. The plants were ready for field transfer in 3 months.

For comparison, mature seeds obtained from dried follicles were soaked in water overnight and sown in soil to raise seedlings under greenhouse environment and leaves were also collected from the middle part of the old branches of mature field grown trees at first forking level.

Abaxial epidermal peels were prepared by treatment of leaves with Jeffery's fluid and stained in safranine, mounted in glycerine and sealed with *DPX*™ mountant (containing dibutyl phthalate, xylol and distrene 80; *Central Drug House*, New Delhi, India) as per the method described by Hussin *et al.* (1997). Fully expanded leaves from the uppermost node collected at different phases of micropropagation *viz.* multiplication, rooting and hardening (all three modes) were used for peel preparation. Controls consisted leaves from *in vivo* raised seedlings (in soil, in greenhouse, 30-d-old), *in vitro* raised seedlings (on 0.8 % water-agar, in aseptic conditions, 30-d-old) and from mature tree of *W. tomentosa*. Twenty leaves from each plant, ten peels from each leaf, five plants from each phase including control were studied for stomatal characteristics. All the parameters were studied from the middle of the leaf within 0.5 mm of the main vein. Various parameters were calculated: stomatal frequency (the number of stomata per unit area), percentage of closed stomata, percentage of malformed stomata. Size of stomata was measured with the help of ocular micrometer pre-calibrated with stage micrometer.

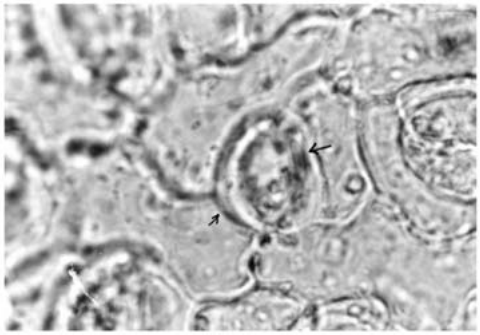

Fig. 1. Abnormal, collapsed, completely closed stomata with malformed guard cells in *in vitro* leaves of *Wrightia tomentosa.*

Detached, approximately same sized leaves (six from five plants each) from various samples (described earlier) were weighed fresh and assayed for rate of water loss. Leaves were allowed to transpire by keeping their abaxial side up on clean bench at room temperature of 25 - 28 °C and RH of 35 - 40 %. Each leaf was weighed at 15 min interval for 3 h. Finally all the leaves were oven dried at 60 °C to obtain dry mass. Water loss was calculated as described by Brainerd and Fuchigami (1981): water loss [%] = [(initial fresh mass- mass after holding)/(fresh mass - dry mass) × 100].

The stomata on leaves collected from mature tree of *W. tomentosa* were paracytic, elliptical, with distinct kidney-shaped guard cells and sinusoidal epidermal cells. Such normal stomata were also noted in the leaves of water-agar-raised seedling and *in vivo* raised seedlings. Qualitative observations in the leaves of multiplication phase cultures revealed malformed stomata. Such stomata were either wide open, with unequal guard cells or with completely collapsed guard cells. These stomata were round with more pore area. Percentage of malformed stomata was 29.4 in multiplication phase and such irregularities, although of reducing degree, could be traced up to rooting (25.8 %) and during *in vitro* hardening phases (12.8 %). No deformity in stomatal morphology was recorded in *in vivo* formed leaves of hardened plants.

The epidermal cells on leaves of multiplication phase cultures were polygonal, having no sinuous undulations in anticlinal walls. Epidermal cells in other phases of micropropagation as well as in controls were more regularly shaped and with many undulations. Stomata on leaves collected from all the phases of micropropagation except multiplication phase were similar in length and width. In leaves of multiplication phase stomata were almost round (18.0 μm long and 16.0 μm wide) with spherical pores as compared to normal bean shaped (21.7 μm long and 13.6 μm) stomata with elliptical pores.

Stomatal frequency was more than double in leaves from multiplying cultures (565 mm^{-2}), compared with

Table 1. Stomatal characteristics of *W. tomentosa* leaves during different phases of micropropagation (means ± SE, $n = 5$).

Source of leaf	Stomatal frequency [mm^{-2}]	Length of stomata [μm]	Width of stomata [μm]
Multiplication phase	565.0 ± 39.2	18.00 ± 0.68	16.02 ± 0.78
Rooting phase	241.0 ± 12.2	21.46 ± 0.96	12.19 ± 0.94
In vitro hardening	566.9 ± 12.1	21.46 ± 0.96	12.19 ± 0.94
In vivo formed leaves	230.2 ± 12.3	21.04 ± 0.52	13.70 ± 0.33
In vitro raised seedlings	402.0 ± 31.2	21.03 ± 0.54	13.72 ± 0.34
In vivo raised seedlings	258.0 ± 12.6	21.68 ± 0.49	13.64 ± 0.53
Mature tree	234.0 ± 13.2	20.03 ± 1.21	14.01 ± 1.02

leaves obtained from *in vivo* raised seedlings (258 mm^{-2}) and mature tree (234 mm^{-2}). The frequency of stomata dropped during rooting phase (241 mm^{-2}) with the broadening of leaf lamina but increased again by 2-fold (566.9 mm^{-2}) in the subsequent *in vitro* hardening phase. Usually found stomatal frequencies were regained during subsequent period, with 230.19 stomata per unit area on leaves developed *in vivo* in open bottles. Similar effect of environment was noted in seedlings. Leaves of *in vitro* raised seedlings exhibited double the stomatal frequency as compared to *in vivo* seedlings.

More than 95 % stomata were open in leaves obtained from cultures (*in vitro* raised seedlings, multiplying cultures and rooted plantlets) and in plants undergoing hardening in closed-cap bottles. Loosening of caps resulted in a slight decline (91.38 %) in number of open stomata. However, after gradual exposure to *ex vitro* environment, stomatal regulation was apparent with only 14.58 % open stomata in leaves formed *in vivo* in hardened plantlets.

A rapid water loss (31.8 %) was noted in leaves from multiplication phase within the first 15 min. The water loss was 13.57 % in leaves of rooted plants while only 2.4 % in *in vivo* raised seedlings and 3.8 % in the leaves of the mature tree. With time, the rate of water loss decreased in each case. Maximum water loss occurred in leaves from multiplying phase with a total of 93.4 % in 3 h. Leaves of *in vivo* raised seedling and those from mature tree exhibited least total water loss (15.2 % and 16.08 %, respectively). *In vitro* hardened (in closed caps) and rooted plants exhibited similar water loss (39.4 and 41.2 %, respectively) in the holding time of 3 h.

After a gradual exposure of plantlets to *ex vitro* conditions, an increasing control on water loss was noted. On complete removal of caps, *in vivo* produced leaves which showed 30.9% total water loss during 3 h. More than 10 % decrease in rate of water loss was observed in leaves obtained after 15 d of cap removal. No further decrease in rate of water loss was observed in subsequent pot transfer.

The survival rate of plants was influenced by the mode of hardening. Plantlets transferred directly to pots in open air could not survive beyond a week. Hardening by covering the pots though increased the longevity of survival time, yet such plants could not survive beyond a fortnight after their gradual exposure to *ex vitro* conditions. *In vitro* hardening proved to be the best procedure for micropropagated *W. tomentosa* plantlets with more than 95 % survival after 3 months.

Desiccation after *ex vitro* transfer is the common cause of mortality in plants raised by tissue culture technique. Stomatal abnormalities and poor epicuticular wax formation are the common causes for mortality of tissue culture plantlets (Sutter and Langhans 1979). However, in some case cuticle may have no role as such (Santamaria and Kerstiens 1994). Stomata account for high transpiration losses and control of transpiration by *in vitro* plants via stomata or leaf epidermis is regarded essential in order to survive *ex vitro* transfer (Van Huylenbroeck and Debergh 1996). In present studies leaves of *in vitro* plants exhibited higher stomatal frequencies than those of *in vivo* plants (mature tree and *in vivo* raised seedling). As was reported in roses (Capellades 1990) and *Delphinium* (Santamaria *et al*. 1993). Wetzstein and Sommer (1982) found significantly higher stomatal densities in culture as compared to field grown or acclimatized plant leaves. Ross-Karstens *et al*. (1998) noted higher stomatal density in the *in vitro* grown grape leaves cultured in closed vessel as compared to those developed *in vivo*. Blanke and Belcher (1989) determined up to 575(stomata) mm^{-2} for apple leaves *in vitro* compared to 390 mm^{-2} grown *in vivo*. In *W. tomentosa*, the leaves of rooting phase and seedlings exhibited stomatal frequencies similar to that found in *Rosa* (Capellades *et al*. 1990). In present studies, stomata on leaves from mature tree, water agar raised seedling

Table 2. Water loss [%] from detached leaves of plantlets during different phases of micropropagation in *W. tomentosa* (means ± SE of five plants).

Time [min]	Multiplication	Rooting	*In vitro* hardened plants	*In vivo* raised seedlings	Mature tree
0 - 15	31.76 ± 5.34	13.75 ± 2.75	6.25 ± 0.88	2.40 ± 0.47	3.82 ± 0.24
16 - 30	13.46 ± 2.45	6.53 ± 0.83	3.11 ± 0.61	2.57 ± 0.43	2.33 ± 0.10
31 - 45	17.34 ± 2.68	5.35 ± 0.77	5.55 ± 0.93	1.15 ± 0.10	2.47 ± 0.11
46 - 60	9.04 ± 1.04	3.03 ± 0.48	4.06 ± 0.81	0.73 ± 0.07	2.34 ± 0.09
61 - 75	7.64 ± 0.91	3.64 ± 0.55	4.09 ± 0.78	0.91 ± 0.06	2.17 ± 0.09
76 - 90	5.03 ± 0.79	1.02 ± 0.09	3.07 ± 0.56	0.91 ± 0.07	0.43 ± 0.02
91 - 105	5.97 ± 0.83	1.31 ± 0.13	2.20 ± 0.19	1.05 ± 0.06	0.60 ± 0.03
106 - 120	0.92 ± 0.08	1.15 ± 0.09	2.61 ± 0.17	0.92 ± 0.07	0.47 ± 0.03
121 - 135	1.02 ± 0.09	1.17 ± 0.16	2.20 ± 0.08	1.05 ± 0.07	0.82 ± 0.04
136 - 150	0.96 ± 0.08	1.31 ± 0.11	2.13 ± 0.10	1.19 ± 0.08	0.31 ± 0.02
151 - 165	0.30 ± 0.03	1.13 ± 0.08	2.29 ± 0.09	1.09 ± 0.10	0.16 ± 0.02
166 - 180	0.00	1.20 ± 0.03	2.01 ± 0.11	1.25 ± 0.09	0.11 ± 0.01
Total	93.44	41.21	39.39	15.23	16.08

and *in vivo* formed leaves of hardened plants were elliptical and depressed while they were large and wide open on leaves of *in vitro* raised plants. This is supported by the investigation in *Liquidamber styracifolia* (Wetzstein and Sommer 1982) where the stomata were superficial, circular and more numerous in leaves of *in vitro* plants while elliptical, depressed and less numerous in the leaves of hardened plants (Tichá *et al*. 1992, Pospíšilová *et al*. 1999). High stomatal frequency in the seedling leaves raised *in vitro* clearly indicated that physical cultural conditions increased number of stomata. Malformed stomata once formed could not be corrected.

Though the stomatal frequencies in leaves of multiplying cultures were similar to those of *in vitro* hardening phase, the former exhibited no control on water loss. The latter lost an amount of water like leaves of rooting phase. Moreover, the stomatal frequency during the hardening phase was double the frequency observed in the rooting phase. The differential behaviour may be correlated with the percentage of malformed stomata. Increased stomatal density in plants grown in closed vessels is in agreement with Woodward and Bazzaz (1988) who found that a CO_2 partial pressure below ambient tends to increase the number of stomata per leaf area. Irregularly shaped epidermal cells, without or with few undulations were seen during multiplication while sinuous undulations were evident during the rooting phase in *W. tomentosa*. Similar observations were recorded by Capellades *et al*. (1990) when *Rosa* cultures were transferred to 75 % RH.

It is suggested that in guard cells of leaves raised *in vitro*, the protoplast could be functional, but the physiological functions related to wall structure and water movement are modified (Ziv *et al*. 1987, Capellades *et al*. 1990, Sallanon *et al*. 1993). Sallanon *et al*. (1993) have concluded that stomata on leaves raised *in vitro* got structural or physiological malfunctioning which could not be repaired. To be functional, stomatal ontogenesis should not experience too high RH. In *Rosa*, it occurred in greenhouse. Present studies strongly confirm their findings. *Ex vitro* hardening did not support *in vivo* leaf formation. The *in vitro* hardening was successful as it provided a sufficient period for gradual exposure of plantlets to external environment. This is in contradiction to observations recorded for apple (Brainerd and Fuchigami 1981, Capellades *et al.* 1990) where short term humidity acclimatization changed stomatal function, and plants could be easily hardened.

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