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Development Note

Histological observation of changes in leaf structure during successive micropropagation stages in *Aralia elata* and *Phellodendron amurense*

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Abstract The micropropagation protocols for *Aralia elata* and *Phellodendron amurense* were established. The survival rates in the acclimatization process between the two species were found to be greatly different. To find the reasons for this difference, the morphological and histological changes in the leaf during successive micropropagation stages in *in vitro*-derived plantlets of *A. elata* and *P. amurense* were compared. Significant differences in mesophyll development in differentiation as well as vascular system development were found during the root induction stages. Such different leaf structures may be responsible for the survival rate through the acclimatization process. These results suggested that changes in leaf structure from *in vitro* to *ex vitro* plants are important for histological development, rendering them appropriate for *in vitro* hardening, which begins at the root elongation stage.

Key words: Acclimatization, *Aralia elata*, *in vitro* hardening, leaf structure, *Phellodendron amurense*.

When plantlets are transplanted from an *in vitro* culture to greenhouse conditions, they often desiccate or wilt rapidly and finally die due to their undeveloped histological structures. Consequently, substantial precautions must be taken after transfer to open air and soil so that plants can overcome sudden environmental changes and acclimatize to new environments (Tichá et al. 1999). To determine how micropropagated plants may best be transferred to normal growth conditions, more information is needed in regard to histological changes during *in vitro* development (Donnelly and Tisdall 1993). Leaves of *in vitro*-plantlets show poor activities in stomatal functioning, epicuticular wax formation, and water transport (Majada et al. 1998; Pospíšilová et al. 2000), resulting in low survival rates during the acclimatization stages. The morphological and histological changes occurring in the leaves of plantlets may be critical during the micropropagation stages. However, information in this regard is limited (Brutti et al. 2002; Apóstolo et al. 2005).

We previously established the micropropagation protocols for *A. elata* and *P. amurense* (Azad et al. 2004a,b, 2005a,b; Azad 2006; Karim et al. 2006, 2007) and found significant differences in the survival rate of two species during acclimatization. *A. elata* plantlets derived from rooted shoots showed a final survival rate of 72%, while *P. amurense* plantlets derived from internode calli showed a 90% survival rate (Azad 2006; Karim et al. 2007).

In the present study, we examined the morphological and histological changes in leaf structures during successive micropropagation stages in *in vitro*-derived plantlets of *A. elata* and *P. amurense* and attempted to increase their survival rate during the acclimatization process.

The culture conditions and periods for shoot induction and multiproliferation, root induction and elongation, and acclimatization of plantlets of *A. elata* and *P. amurense* are shown in Table 1. The culture and acclimatization conditions, combination of plant growth regulators (PGRs) in suitable basal media for shoot induction and multiplication, and root induction and

Abbreviations: BAP, 6-Benzyl aminopurine; BT, broad-leaved tree medium (Chalupa 1984); IBA, indole-3-butyric acid; IS, intercellular space; LA, leaf area; LE, lower epidermis; LT, leaf thickness; MS, Murashige and Skoog (1962) medium; MX; metaxylem; NAA, α-naphthaleneacetic acid; PC, palisade cell; PGR, plant growth regulator; PH, phloem; PP, palisade parenchyma; PS, paliside parenchyma; SC, spongy cell; SP, spongy parenchyma; UE, upper epidermis; XY, xylem.
This article can be found at http://www.jspcmb.jp/

Table 1. Experimental design for the histological observation of plantlet leaf leaves in *A. elata* and *P. amurense* during successive micropropagation stages

Period (week)	Stage	Medium and PGRs		Note
		A. elata	*P. amurense*	
0	Shoot induction and multiproliferation	BT+0.5 μM BAP	MS+2.0 μM BAP+ 0.5 μM NAA	Nodal segments were planted.
1				
2				
3				
4				Leaf was collected (M1 stage).
5				
6				
7				Leaf was collected (M2 stage).
8	Root induction and elongation	BT+2.0 μM NAA	MS+2.0 μM IBA	Leaf was collected (R1 stage).
9				
10				
11				
12				Leaf was collected (R2 stage).
13	Acclimatization	Vermiculite		Leaf was collected (A1 stage).
14				
15				
16				
17				Leaf was collected (A2 stage).
18		Soil		
19				
20				
21				
22				
23				Leaf was collected (A3 stage).

BT, Broad-leaved tree medium (Chalupa 1984); MS, Murashige and Skoog medium (Murashige and Skoog 1962); BAP, 6-benzyl aminopurine; NAA, α-naphyalene acetic acid; IBA, indole-3-butyric acid; M1, shoot multiproliferation stage; M2, pre-rooting stage; R1, root induction stage; R2, root elongation stage; A1 and A2, acclimataization stages, with persistent leaves 1 week (A1) and 4–6 weeks (A2) after transfer to vermiculite; A3, acclimataization stage on soil with *ex vitro* new leaves onto soil.

elongation were previously described (Azad 2006; Karim et al. 2007). Leaves were collected from *A. elata* and *P. amurense* plantlets at 7 different micropropagation stages (Table 1) according to the published methods (Brutti et al. 2002), which defined M1 as the shoot multiproliferation stage, M2, the pre-rooting stage, R1, the root induction stage, R2, the root elongation stage, A1 and A2, the acclimatization stages with persistent leaves 1 week (A1) and 4–6 weeks (A2) after transfer to vermiculite, respectively, and A3, the acclimatization stage on soil with *ex vitro* new leaves. For the histological observation, the collected leaves were cut into 5 mm^2 pieces from the middle portion with the mid-rib and then fixed, embedded, cut, and stained by the method described (Karim et al. 2006). A histological observation was also conducted according to a previously described method (Karim et al. 2006). In the present study, the following items were measured: leaf area (LA), leaf thickness (LT), palisade parenchyma (PP) thickness, spongy parenchyma (SP) thickness, width and length of the palisade cell (PC), and spongy cell (SC). These items, except for LT, were measured using images obtained with a microscope (BX51, Olympus, Japan) and a digital camera (DP11, Olympus, Japan). The images were analyzed with a computer and Scion image software (Scion Corp., USA). For statistical analysis, three leaves were selected for each healthy plantlet, and three sections were taken from each leaf; morphological measurements were repeated three times. A total of 45 individuals per parameter were measured. A statistical analysis was conducted by Tukey's multiple comparison test with JMP Statistical Discovery Software (SAS Institute, USA) for each individual character; the analysis showed significant variation among different successive growth stages.

Changes in the morphological and histological features of leaves from *A. elata* plantlets were observed at different growth stages (M1–A3) (Table 2). Samples at the M1 and A3 stages showed the lowest (9.0 mm^2) and the highest (286.2 mm^2) LA, respectively. A histological observation of leaves at different developmental stages revealed that leaves at the M1 stage had the lowest LT (40.7 μm), with an SP/PP ratio of 1.9. At the M2 stage, the SP/PP ratio was also 1.9. The SP/PP ratios of R1, R2, A1, A2, and A3 were 1.2, 1.3, 1.4, 1.2, and 1.2, respectively. At these stages (R1–A3), the SP/PP ratio was almost the same, whereas the cell size and arrangement were different in pattern; the PP thickness gradually increased, as did the LT, although the SP thickness did not show a definite trend. This may be due

Table 2. Morphological and histological observation of *in vitro A. elata* leaves during successive micropropagation stages

Items	Stage						
	M1	M2	R1	R2	A1	A2	A3
LA (mm^2)	9.0 g	16.0 f	27.3 e	79.6 d	99.4 c	166.3 b	286.2 a
LT (μm)	40.7 f	55.0 d	46.4 e	58.7 d	62.7 c	85.1 b	98.1 a
PP thickness (μm)	10.4 d	14.9 c	15.2 c	19.8 b	16.9 c	22.4 b	25.0 a
SP thickness (μm)	20.4 d	29.3 a	18.7 d	26.8 b	24.0 c	27.6 b	30.8 a
PC width (μm)	3.6 e	5.6 d	5.8 d	7.0 c	7.0 c	8.8 b	11.2 a
PC length (μm)	7.3 d	13.0 c	12.6 c	18.5 b	14.3 c	20.6 a	22.0 a
SC width (μm)	4.0 d	5.8 c	5.9 c	6.9 c	6.8 c	9.5 b	12.2 a
SC length (μm)	6.7 e	9.6 d	10.7 d	10.3 d	14.9 c	18.5 b	22.7 a
SP/PP ratio	1.9	1.9	1.2	1.3	1.4	1.2	1.2

LA, leaf area; LT, leaf thickness; PP, palisade parenchyma; SP, spongy parenchyma; PC, palisade cell; SC, spongy cell; M1, shoot multiplication stage; M2, pre-rooting stage; R1, root induction stage; R2, root elongation stage; A1 and A2, acclimatization stages, with persistent leaves 1 week (A1) and 4–6 weeks (A2) after transfer to vermiculite; A3, acclimatization stage on soil with *ex vitro* new leaves. Means followed by the same letter are not significantly different by Tukey's multiple range test at 0.05 probability.

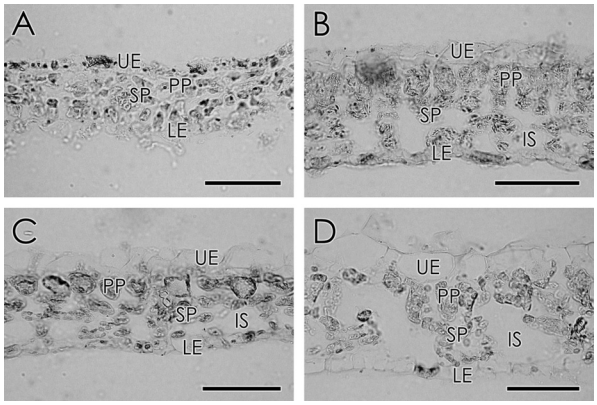

Figure 1. Development of the leaf structure of *A. elata* plantlets during successive micropropagation stages. A: M1 stage, shoot multiproliferation stage; B, R2 stage, root elongation stage; C, A2 stage, acclimatization stage, with persistent leaves 4–6 weeks after transfer to vermiculite; D, A3 stage, acclimatization stage on soil with *ex vitro* new leaves; UE, upper epidermis; PP, palisade parenchyma; SP, spongy parenchyma; LE, lower epidermis; IS, intercellular spaces; scale bar=50 μm.

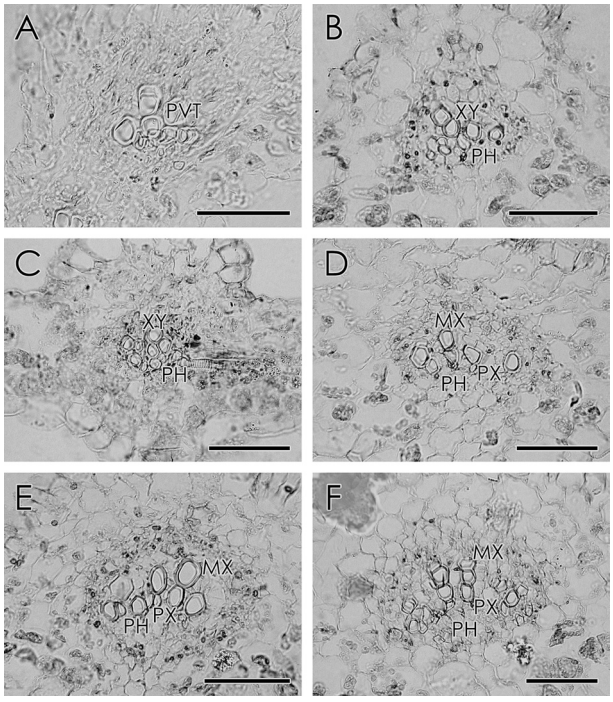

Figure 2. Development of vascular bundle of *A. elata* plantlets during successive micropropagation stages. A, M1 stage; B, R1 stage, root induction stage; C, R2 stage; D, A1 stage, acclimatization stage, with persistent leaves 1 week after transfer to vermiculite; E, A2 stage; F, A3 stage; PVT, provascular tissue; XY, xylem; PH, phloem; MX, metaxylem; PX, protoxylem; scale bar=50 μm.

to large changes in the distribution and size of the intercellular spaces (IS). As a result, the primitive stages (M1 and M2) of leaf development had a relatively high SP/PP ratio, while the advanced stages (R1–A3) had relatively small values. Of all growth stages, the acclimatization (A3) stage had the greatest leaf thickness, 98.1 μm. The mesophyll cells consisted of two types of parenchyma cells, palisade and spongy. At the M1, M2, and R1 stages, the PP and SP were composed of more irregularly shaped cells arranged in a loose structure (Figure 1A). The mesophyll cells at these stages were not well arranged and organized. The thickness of the adaxial epidermis (UE) was greater than that of the abaxial epidermis (LE). These epidermises gradually developed at the advanced stages. At these stages, the IS were rare and small in size (Figure 1A). Vascular connections were poorly developed at the M1 and R1 stages (Figure 2A, B). During the root elongation stage (R2), a more organized structure was observed (Figure 1B) in mesophyll tissues with large IS in the abaxial side. The mesophyll consisted of one PP layer and 3–4 SP layers. The arrangement of the PP was comparatively organized relative to those at the M1 and M2 stages. Vascular connections were well developed and comparatively differentiated (Figure 2C). Well-developed mesophyll cells were observed in the leaves at the *ex vitro* stages (A1–A3). The mesophyll was composed of one PP layer and 3–4 SP layers (Figure 1C, D) and a higher number of SP layers at the more developed stage (A3), in which the PP and SP cells were

Table 3. Morphological and histological observation of *in vitro P. amurense* leaves during successive micropropagation stages

Items	Stage						
	M1	M2	R1	R2	A1	A2	A3
LA (mm^2)	15.4 e	23.3 e	42.4 d	79.0 c	101.8 b	203.2 a	209.7 a
LT (μm)	157.9 a	111.7 c	97.4 f	104.6 d	105.9 d	101.2 e	123.1 b
PP thickness (μm)	42.1 b	30.7 d	29.3 d	35.2 c	34.3 c	35.7 c	50.9 a
SP thickness (μm)	81.5 a	45.1 c	35.7 e	40.3 d	37.7 e	36.6 e	49.8 b
PC width (μm)	20.6 a	12.7 c	10.8 d	14.9 b	11.4 d	11.5 d	9.5 e
PC length (μm)	4.9 b	31.4 d	27.2 f	29.8 e	34.2 c	34.9 c	47.1 a
SC width (μm)	22.2 a	13.1 c	10.5 d	14.1 b	10.9 d	11.7 d	10.3 d
SC length (μm)	31.1 a	24.3 c	21.1 d	26.8 b	19.4 e	23.5 c	21.6 d
SP/PP ratio	1.9	1.4	1.2	1.1	1.0	1.0	1.0

LA, leaf area; LT, leaf thickness; PP, palisade parenchyma; SP, spongy parenchyma; PC, palisade cell; SC, spongy cell; M1, shoot multiplication stage; M2, pre-rooting stage; R1, root induction stage; R2, root elongation stage; A1 and A2, acclimatization stages, with persistent leaves 1 week (A1) and 4–6 weeks (A2) after transfer to vermiculite; A3, acclimatization stage on soil with *ex vitro* new leaves. Means followed by the same letter are not significantly different by Tukey's multiple range test at 0.05 probability.

poorly arranged. The UE at the A3 stage was wider than those at other stages. Among three sections of acclimatization stages, large ISs were present at the A3 stage. The vascular systems at these stages were very well developed and distributed separately, and the xylem (XY) tissues were properly arranged in a line in the sequence of the metaxylem (MX) to the protoxylem (PX) from the adaxial side to the abaxial side (Figure 2 D, E, F). The A3 stage showed the most organized vascular structure of all other stages, and each XY tissue was separately arranged.

The morphological and histological features of leaves from *P. amurense* gradually changed throughout the growth stages from M1 through A3 (Table 3). Of the different stages, the M1 had the smallest LA (15.4 mm^2), and the A3 had the largest one (209.7 mm^2), indicating that the LA gradually increased from the M1 to the A3 stages. Some variations in the LT were observed among the different stages. The leaves in the M1 stage had the highest LT. In the leaves at the M1 stage, the SP thickness was almost twice that of the PP. At the M2 stage, the SP/PP ratio was 1.4. This ratio gradually decreased until the A1 stage. On the other hand, the leaves at the A1–A3 stages showed the same ratio, 1.0. At the primitive stages (M1, M2, and R1), the PP was composed of oblong, upright cells, and the SP consisted of more irregularly shaped cells arranged in a loose structure (Figure 3A). The UE was large and regularly arranged, with generally straight anticlinal walls. The LE was smaller and resembled adjacent mesophyll cells. Vascular connections were small and poorly developed at the M1 and M2 stages (Figure 4A, B). The vascular bundles at the R1 stage showed better tissue organization than those at the M1 and M2 stages (Figure 4C). At the root elongation stage (R2), a more organized mesophyll structure was observed with a greater number of cell layers (Figure 3B). Larger and more prominent vascular bundles were observed at this stage (Figure 4D). The leaves at the *ex vitro* (A1–A3) stages showed a well-

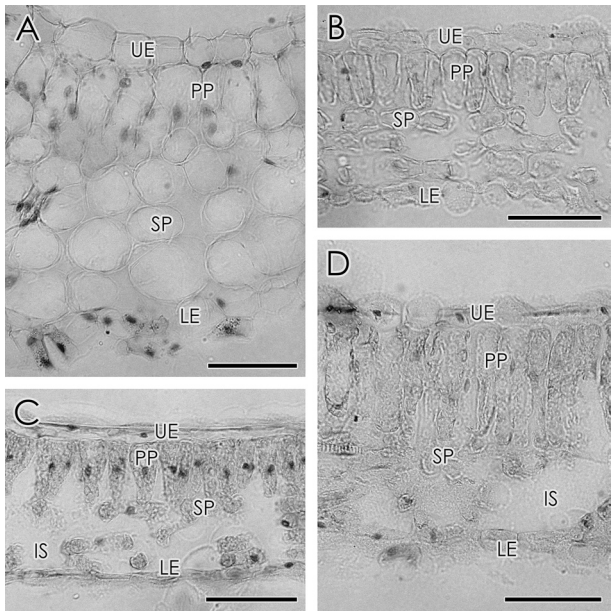

Figure 3. Development of the leaf structure of *P. amurense* plantlets during successive micropropagation stages. A, M1 stage; B, R2 stage; C, A2 stage; D, A3 stage; UE, upper epidermis; PP, palisade parenchyma; SP, spongy parenchyma; LE, lower epidermis; IS, intercellular spaces; scale bar = 50 μm.

defined mesophyll, in which the PP cells were typically rectangular and arranged in one layer, and showed a very compact, regular, and continuous distribution; the SP was composed of 4–5 layers of cells with large IS (Figure 3C, D). The PP cells were longer than those at the *in vitro* stages. The vascular system developed very well at these stages (Figure 4E, F, G). The XY was formed on the adaxial side of the vascular bundle, while the phloem (PH) was on the abaxial side.

Some differences in leaf histology during cell development within a leaf were found between *A. elata* and *P. amurense* (Tables 2 and 3). The leaf thickness was smaller in *A. elata* than in *P. amurense*, whereas both the PP and SP were thicker in *P. amurense* than in *A. elata*.

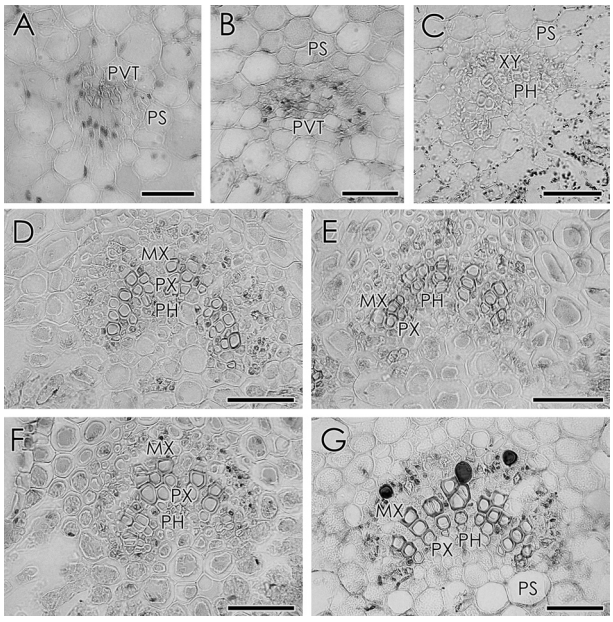

Figure 4. Development of the vascular bundle of *P. amurense* plantlets during successive micropropagation stages. A, M1 stage; B, M2 stage; C, R1 stage; D, R2 stage; E, A1 stage; F, A2 stage; G, A3 stage; PVT, provascular tissue; PS, parenchyma sheath; XY, xylem; PH, phloem; MX, metaxylem; PX, protoxylem; scale bar=50 μm.

The PP cells of *P. amurense* were better arranged and longer than those of *A. elata* as leaf development proceeded. On the other hand, the UE and LE of *A. elata* were wider than those of *P. amurense*. *P. amurense* leaves showed more organized vascular connections than those of *A. elata*.

Notable changes in leaf histology were observed during different *in vitro* stages as well as after transferring plantlets to soil. The leaf size increased gradually during the micropropagation stages (M1–A3) in both *A. elata* and *P. amurense* (Tables 2 and 3). Similar observations were noted by Brutti et al. (2002) and Apóstolo et al. (2005) in *Cynara scolymus* L. leaves. *In vitro* leaves tended to have one layer of PP and three layers of SP. The present study showed that one layer of PP and three layers of SP were present at the *in vitro* stages of *A. elata* as well as *P. amurense*.

The leaves of *P. amurense* from the R2 to A3 stages had distinct differentiation of the mesophyll tissues into PP and SP with high cell density and large IS (Figure 3B, D). At the R2 stage, a more organized mesophyll than that at previous stages was observed (Figure 3A, B). Louro et al. (1999) reported that there were more structural differences in the mesophyll of *in vitro Eucalyptus* leaves during the root elongation stage than during the multiplication stage. The PP cells gradually increased in number and size. This observation reinforces the idea that alterations in the PP cells influenced the photosynthetic system and the low rate of photosynthesis in *in vitro* plants (Smith et al. 1986). Louro et al. (1999) concluded that morphological changes in PP cells are involved in the higher photosynthetic capacity and the increase in metabolic activity required for leaf expansion during root formation.

The most important changes were found during mesophyll development and vascular system development, both of which were observed at the root induction stages of *A. elata* and *P. amurense*. These changes were possibly caused by the presence of auxins in the rooting media. At the root elongation stage, auxins enhanced the growth and development of roots as well as the shoots of plantlets. Similar results were reported in *Malus pumila* plantlets during acclimatization (Diaz-Pérez et al. 1995).

The development of a vascular bundle in leaves from the R2 to A3 stages of *A. elata* and *P. amurense* was examined in this study. At these stages, a vascular bundle developed gradually, and XY was specified as PX and MX (Figure 4E, F, G). Similar results were observed in *Vaccinium corymbosum* (Noé and Bonini 1996) and *Cynara scolymus* (Apóstolo et al. 2005) leaves. A developed vascular bundle might enhance the acclimatization of plantlets to form a water- and nutrient-uptake system.

The structural changes observed in *A. elata* and *P. amurense* leaves from *in vitro* to *ex vitro* plants indicate that histological differences account for some of the procedural changes that are necessary for the hardening of these plants. These differences begin at the root induction/elongation stage. Our findings in this study also suggest that acclimatization will be improved by auxin action on root development. Since the morphology and histology of leaves change in a root elongation medium, such changes may result in a higher survival rate of transplants.

The present findings suggest that differences in the survival rate of two species may be attributable to differences in the development of the mesophyll layer, especially in PP cells, and to differences in the development of the vascular connection. The successful acclimatization of plantlets may require the full development of leaves, and this may largely depend on the rooting stages. This means that *in vitro* hardening is important. In the present study, we attempted to determine the anatomical changes in leaves undergoing acclimatization and to select better-suited plants for micropropagation.

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