Plant Growth Regulation **20**: 17–23, 1996.

General paper

A Study of the graft union in *in vitro* micrografted apple

Ffiona V.M. Richardson[1], Seán Mac An tSaoir[1,2,*] & Barbara M.R. Harvey[1]
[1]*Department of Applied Plant Science, The Queen's University of Belfast, Newforge Lane, Belfast, UK;*
[2]*Department of Agriculture for Northern Ireland, Horticultural and Plant Breeding Station (NIHPBS), Manor House, Loughgall, BT61 8JB, UK (*author for correspondence)*

Key words: Bramley's Seedling, MM106, M9, M25, *in vitro* grafting, graft union formation, vascular regeneration

Abstract

Light microscopy was used to study graft union formation in *in vitro* micrografts of tissue cultured apple (*Malus domestica*. Borkh). Micrografts were constructed using horizontal incisions to form the grafting surface, and placing the cut ends of rootstock and scion into sterile silicon tubing to permit graft formation to occur.

The outer morphological and histological development was similar for different stock-scion micrograft combinations but graft union formation was slower in heterografts than in autografts. Initial leaf expansion at the scion shoot apex occurred in all micrografted plantlets within 1–4 days and was not indicative of graft success. Progressive scion growth and development could be used as an indication of graft success by ten to fourteen days after grafting and probably was related to establishment of cell to cell contact at the graft interface. Microscopy showed initiation of callus proliferation in the vascular cambium and the pith ten days after grafting. Differentiation was observed subsequently and this was reflected in scion development. Longitudinally orientated cambial cells began to differentiate between twenty and forty days after grafting, and formed a bridge between the vascular cylinders of scion and rootstock. The scions at this stage had as many as eight newly expanded leaves and micrografts were strong enough to permit silicon sleeve removal without damage. Continuity of new vascular elements in rootstock and scion was established around forty days. New vascular elements curved slightly in towards the pith to form a 'c' shaped bridge across the graft union. Vascular development continued until it reached completion after six months.

Introduction

Grafting is an ancient practice which has been documented numerous times throughout history [7]. It is a technique in which two genetically distinct pieces of tissue are brought together and then continue to grow as an individual plant [22]. In modern day plant propagation there are many different methods of grafting [4]. Although the reasons for grafting are numerous [11], the fundamental principles are the same for example, a rootstock provides a disease free rooting base for an 'elite' scion which, due to its genetic constitution and mature status, generally cannot be propagated appropriately by other means [22].

A relatively new concept of grafting known as shoot tip grafting or *in vitro* micrografting, was initially developed [16] and later refined [17] for the production of virus free citrus fruit trees. The technique has subsequently been refined many times [9], and successfully applied to temperate fruit tree production [1, 5, 8], as well as for the production of forest tree species through rejuvenation [14]. *In vitro* grafting has many advantages over conventional plant propagation techniques, and has now been extended to have many other applications [18]. Grafting *in vitro* is a procedure which combines all the advantages obtained from grafting with those of micropropagation [2], which ultimately makes it superior to conventional grafting.

In this study conventional light microscopy has been used to examine graft union development in *in vitro* grafted apple. The scion cultivar Bramley's Seedling, and rootstocks M9 (dwarfing), MM106 (semi dwarfing) and M25 (vigorous) were used. Rootstocks with different effects on scion vigour were used in the study to examine possible differences in graft union formation for different micrograft combinations.

Materials and methods

Scion material was obtained by taking dormant buds from one year old grafted Bramley's Seedling plants. The buds were surface sterilised by immersion in 90% ethanol for ten seconds, followed by a 15 min. treatment with a 10% solution of commercial sodium hypochlorite (*Domestos*, Lever Bros. Ltd.), and three rinses in sterile distilled water. Following excision, meristems were established on MS salts [15] with 5 μM 6-benzylaminopurine (BA), 3.0% (w/v) sorbitol, pH 5.5, for establishment [26]. After four weeks the meristems were transferred onto *Malus* multiplication medium (which is based on MS salts [15] with 3.9 μM BA, 0.5 μM indole-3-butyric acid (IBA), and 3.0% sorbitol; pH 5.5) for a further four to six weeks. A range of tissue cultured rootstocks were provided by HRI, East Malling. All shoots cultured on *Malus* multiplication medium were ready to micrograft between four to six weeks after transfer.

Grafting

Rootstock and scion partners of similar diameter were selected to optimise potential cambial contact. The rootstocks were decapitated and defoliated. In order to reduce desiccation and for ease of manual manipulation the scions were also defoliated taking care not to damage the apex. To perform the graft incision the graft partners were lined up with their cut surfaces parallel, and a horizontal slice was cut from each to expose the graft surface. Autoclaved silicon tubing of suitable internal diameter was used to hold the graft partners in position as the graft union was formed [19, 20].

Autografts of Bramley's Seedling and heterografts of Bramley on rootstocks M9, MM106 and M25 were constructed so that grafts were at a range of developmental stages when sampled for microscopy. Constructed grafts were cultured on hormone free MS salts with 7.5% sucrose [16] pH5.5, and solidified with 7 g l^{-1} agar (Oxoid purified agar). The grafts were put into media ensuring that the silicon tubing was above the agar in order to prevent scion rooting, and cultured in at an irradiance of 175 W/m^2. Axillary shoots that developed on the rootstocks were removed.

Light microscopy

For each scion/rootstock combination five apparently successful grafts were selected at 7, 10, 20, 30, 40, 60, 90 and 180 days after grafting. Strength of the graft union was noted (assessed by ability of the grafts to remain intact) when the silicon tubing was removed and 10, 20, 40, 90 and 180 day old grafts were prepared for microscopy. Specimens were trimmed down to 5mm above and below the graft union (a diagonal cut marked the scion end) after removal of the silicon tubing. They were fixed in 4% glutaraldehyde and 0.25M phosphate buffer overnight, dehydrated through a series of alcohol dilutions (30%; 50%; 70%; 90%, 100% and three times in absolute alcohol for four to five hours per stage), and infiltrated in LR White resin (London Resin Co.) for one week. Polymerisation of the resin was at 60 °C in an oven back filled with argon for 24 hours. Sections 1–10 μm thick were cut using glass knives on a Riechart microtome, and stained with 4% Toluidine blue for ten minutes.

Results

Between one and four days after grafting apical foliar development occurred on all grafts. Graft failure, as indicated by progressive desiccation of the scion, could be seen after 10 d. In contrast, successful grafts continued to grow and develop as the graft union formed between the partners.

When tubing was removed after 7 d all grafts did not remain intact, although expansion and development of new scion foliage was apparent and the scions appeared to be in a good state of health. Ten-day-old grafts remained intact when the tubing was removed, but would fall apart if slight pressure was applied. At this stage the graft surfaces of the partners had undergone little shrinkage, and callus proliferation could be seen with the naked eye as a white circle around the cambium. It was more common for heterografts to fall apart at this stage than autografts. After 20 d. all apparently successful grafts remained intact after removal of

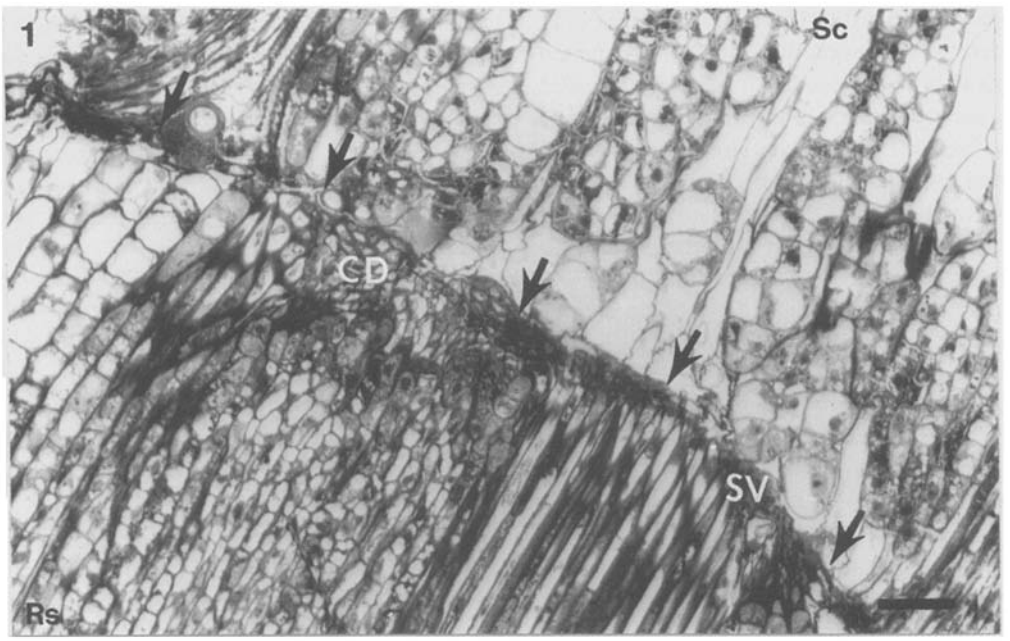

Plate 1. A longitudinal section through a 10-day-old micrograft of Bramley (**Sc**) on M9 (**Rs**) showing necrotic layer (arrows), and areas of cell division (**CD**). Sealed vessels (**SV**) can also be seen. Bar represents 250 μm.

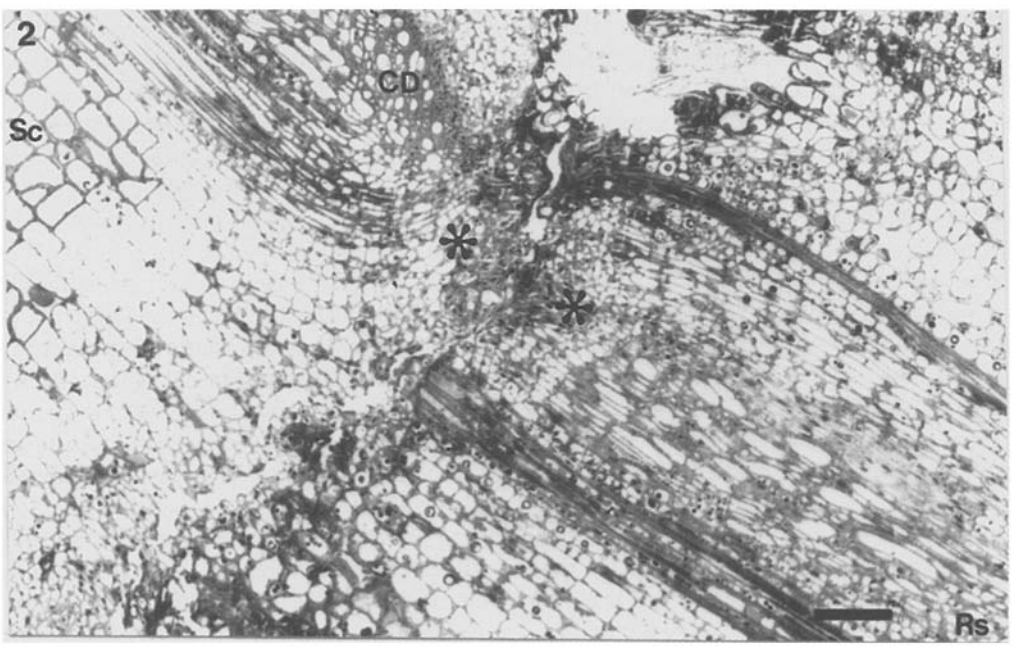

Plate 2. Longitudinal section through a 10-day-old micrograft of Bramley (**Sc**) on M9 (**Rs**) showing callus proliferation (*) and further cell division (**CD**) in areas of the vascular cambium. Bar represents 500 μm.

the tubing, but were still not strong enough to tolerate physical handling. After thirty days the grafted plants could tolerate careful handling without breaking.

Histology of graft development

The histology of graft formation was similar in different graft combinations. During the early stages after

grafting, cambial activity was not obvious. The only visible feature was a distinct layer of heavily stained cells which had been damaged by the blade. 10 d. after grafting the necrotic zone was still visible as a layer of crushed and shrunken cells (Plate 1). In epidermal cell areas there was still no sign of regeneration, although changes were apparent in the vascular tissues. Wound repair had occurred in the damaged vascular strands, sealing the ends of the cut vessels. Cell division had been initiated in the vascular cambium (Plate 2), and the cytoplasm of these cells stained more densely than surrounding cells. Some cells had already undergone cell wall thickening and lignification. These cells were in the process of differentiating into new secondary xylem (Plate 2). Cell division was also occurring in cells along the graft interface, above and below the necrotic zone in the area of the vascular cambium in both graft partners. In some grafts this appeared to cause slight swelling of the tissues close to the interface.

After 20 d. there was a lot of cellular activity at the graft interface as indicated by profuse callus proliferation across the graft union. The necrotic layer had almost disappeared, and the graft union area comprised a multi-layer of callus cells originating from both graft partners (Plate 3). Cortical and pithy parenchyma cells had divided to produce callus cells which were smaller than those at the vascular area. In addition to the massive proliferation of callus, some vascular cambium cell divisions produced elongated cells orientated towards the graft union (not shown). More cells could be seen with secondary cell wall thickening and cell contents became less densely stained as the cells differentiated into new vessels and tracheids. These new vascular elements assumed a longitudinal orientation in both graft partners. There was also some differentiation in the inner pithy area, but the tracheal elements formed in this zone lacked the longitudinal orientation seen in the vascular bundles. This extra- vascular element formation was not seen in cortical areas, but seemed to be restricted to the inner pithy areas.

After 40 d the vascular strands, in particular new vessels and tracheids, could be seen to unite across the graft zone (Plate 4). At this stage there was no trace of the necrotic zone. The numbers of new vascular elements crossing the graft union were fewer than the numbers of old elements in the rootstock and scion, and the connections often meandered rather than having a vertical orientation (Plate 4).

90 d after grafting, cell division and differentiation was still occurring at the graft union but on a smaller scale. Swelling due to further cell division in the vascular cambium could be seen where vascular strands had been severed at the time of grafting. In most autografts it was impossible to identify the exact position of the original graft interface as the wound repair mechanisms were almost complete. Cells in the area stained less densely indicating they had reverted to their normal metabolic state. Evidence of a functional graft union was seen as the realignment of vascular tissue where cambial cells had differentiated to bridge the union as a 'c' shape, reuniting the vascular cylinders of rootstock and scion. The newly formed xylem and phloem were orientated longitudinally, but a few isolated tracheary elements which had differentiated from cells outside the vascular bundles persisted and still lacked longitudinal orientation. The epidermis of the stock and scion tissues had become continuous.

In 90 d old heterografts the protoplasm and nuclear areas of cells in the differentiating region were still densely stained and enlarged. The vascular cambium was still active and produced differentiating cells which reinforced those already crossing the graft union. After 180 d. the process of cell differentiation had slowed down greatly in both autografts and heterografts, but there were still considerably more active cambial cells in the area of the graft union than

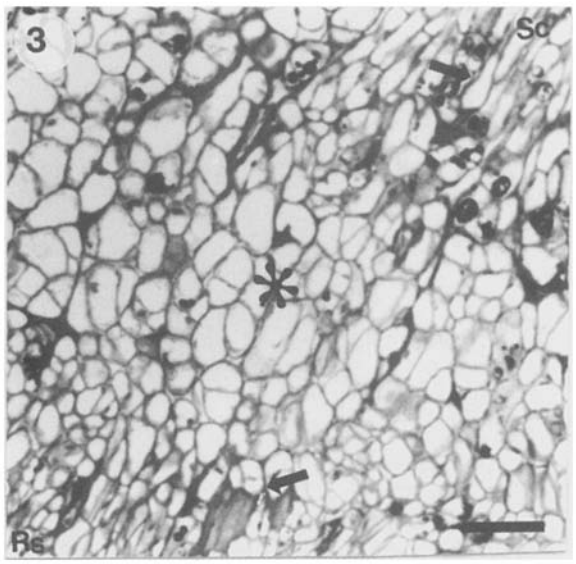

Plate 3. Longitudinal section through a 20-day-old micrograft of Bramley (**Sc**) on MM106 (**Rs**) graft showing callus proliferation (*) connecting graft partners and forming a graft union. Original vascular tissues of the rootstock and scion are indicated by the lower and upper arrows respectively. Bar represents 333 μm.

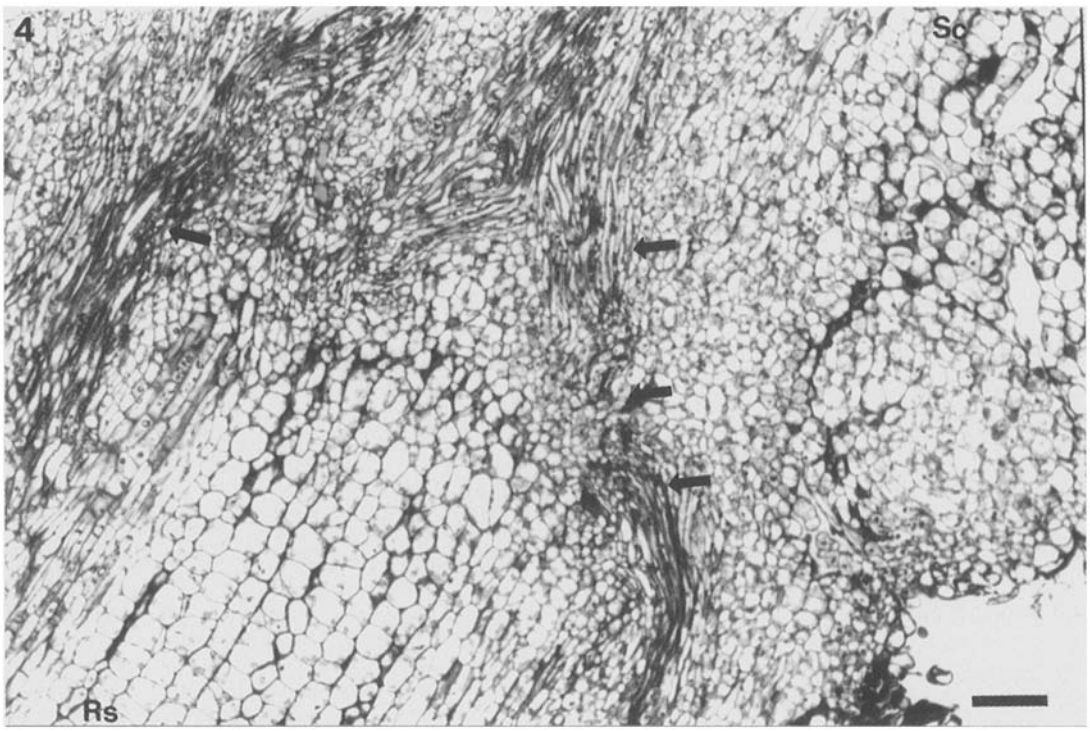

Plate 4. A longitudinal section of a forty-day-old micrograft of Bramley (**Sc**) on MM106 (**Rs**) showing where new vascular elements (arrows) connect graft partners. Bar represents 500 μm.

in the scion and rootstocks stems remote from the graft union (Plate 5).

Discussion

Visible symptoms of graft failure or delayed graft union formation are generally expressed in the scion. However symptoms did not develop immediately under the *in vitro* conditions used for apple in this study. The process of apical foliar expansion which occurred between one and four days after grafting was independent of graft success. Origin of the water necessary to support this process of leaf expansion is debatable. It seems unlikely that the scion's internal reserves would be adequate to support this, despite high humidity in the culture vessels. It is possible that the initial scion leaf expansion occurred due to uptake of xylem sap exuded by the decapitated rootstock plantlet. This exudate would stop after a few days and the scions internal nutrient reserves would then be important as *in vitro* plants have low photosynthetic capabilities [21]. Subsequent scion deterioration in micrografts indicated failure, probably due to insufficient cellular contact between rootstock and scion for water and nutrient movement and inadequate scion reserves to support cell proliferation at the cut surface. Before the scion can become dependent on the rootstock, cellular contact must be established to enable the formation of a symplastic and apoplastic transport system between graft partners.

The ability of micrografts to mechanically support themselves is also important. The mechanical strength of a graft is inherent with the build up a network of cells and new vascular tissues which establish direct contact between stock and scion. After 10 d micrografts fell apart if pressure was applied when the silicon tubing was removed. At this stage there was little direct cell to cell contact because of the presence of a necrotic layer. It is therefore likely that graft partners are held together at this stage by forces of cohesion and adhesion possibly through spillage of damaged cell contents [13]. After 20 d the necrotic layer had dissipated. Cell to cell contact had been established at the interface, and division and differentiation had been initiated. This resulted in the grafts gaining mechanical support and after only 30 d apple micrografts were sufficiently robust to be handled without exercising extreme care.

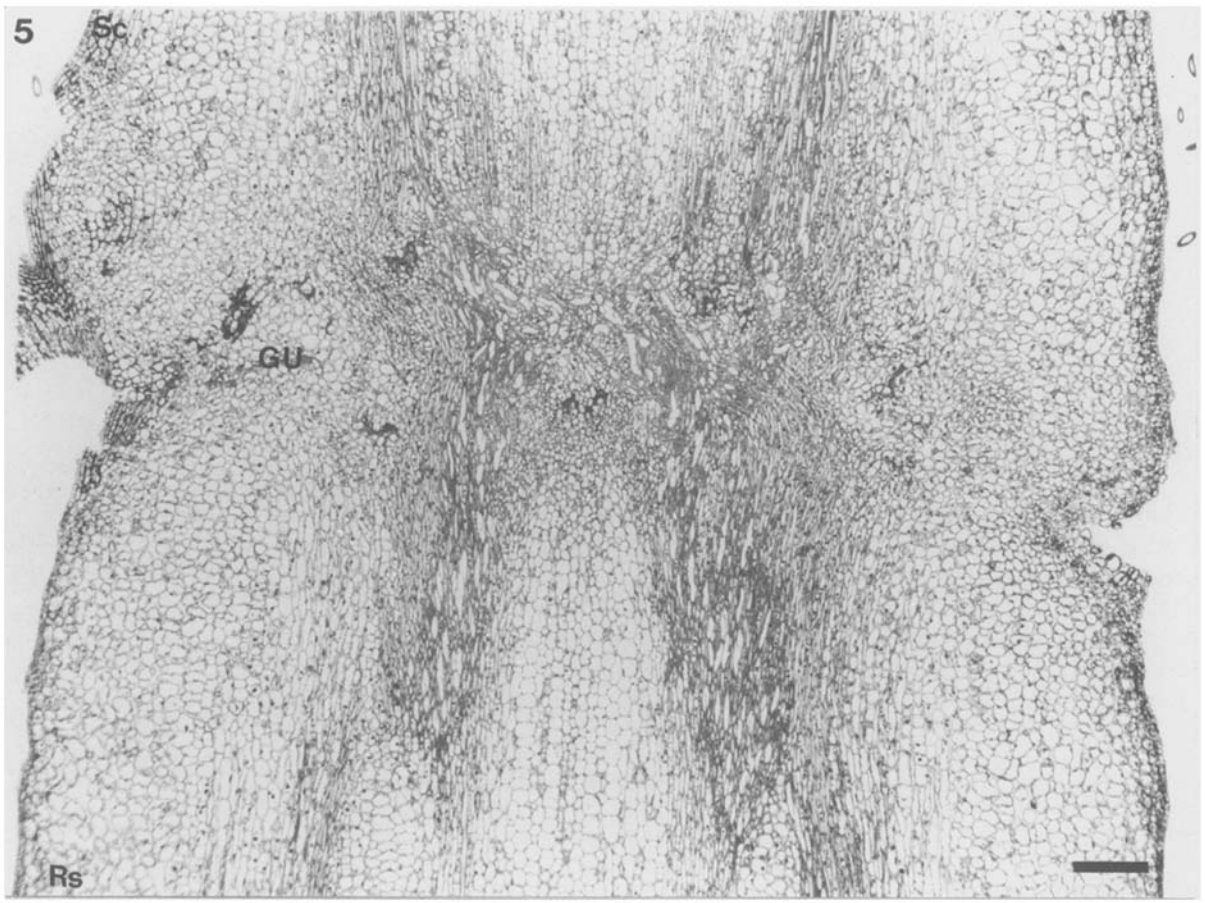

Plate 5. A longitudinal section showing continuity of a vascular system across the graft union (**GU**) in a six-month-old autografted Bramley. (**Rs** = rootstock; **Sc** = scion). Bar represents 800 μm.

Thus by 30 d the grafted plantlets may be physically strong enough to be transferred to the glasshouse for weaning. However their survival would depend upon whether or not the developing vascular system would function adequately to enable the plantlets to withstand the stresses of the glasshouse environment.

From the histological study of graft formation of these micrografts, it can be seen that new vascular elements which interconnect rootstock and scion were present at forty days after grafting, but they were not fully developed. The differentiation process continued for several months. This indicates that although the graft union is physically strong at four weeks, it may not be functionally active until considerably later. Even at 40 d many vascular elements were observed to meander across the graft union. This phenomenon has been documented for conventional grafting [23] where there is misalignment of scion and rootstock vascular tissues but with apple micrografts it occurred even where there was no misalignment. It has been proposed that the pattern of differentiation depends on diffusable substances which promote graft development emanating from the severed vascular bundles [13]. Another possible cause for this meandering pathway could be due to inhibitory effects of compounds such as phenolics released from residual patches of necrotic tissue. These could act as negative influences on cambial development, thus influencing the subsequent pattern of vascular differentiation. The functional efficacy of these meandering vascular connections is also uncertain. A graft can only be described as successful when a fully functional vascular system has been established between the graft partners [6, 25]. Translocation studies, involving ^{14}C-labeling techniques, are underway to assess when apoplastic and symplastic pathways of new vascular systems become functional

and just how efficient they are. These should provide a reliable indicator of the earliest time at which micrografts can safely be transferred to soil. This seems unlikely to depend on the rootstock used because graft development differed little between heterografts with different rootstocks, regardless of their vigour although it was consistently slower with heterografts than autografts as has been documented in other studies [24].

The histological sequence of events in apple micrografts was similar to other accounts of graft formation [12], although the time scale for completion of the union was shorter than for conventional grafting of woody species [3, 10, 22]. Apical meristem grafts of apple [8] and micrografts of *Prunus* [5] have also been reported to form a graft union rapidly. In herbaceous species the process is even more rapid [13]. It is possible that the physiological state of micropropagated apple is more similar to herbaceous plants than to conventional propagation material of woody species.

In conclusion this histological study indicates that union formation *in vitro* is comparable to conventional grafting, but is quite rapid in micrografted apple. Thus *in vitro* techniques previously used only on a small scale for production of virus free apple scion material [1, 8] have potential for large scale production of apple scions micrografted onto commercial rootstocks.

Acknowledgements

This research was funded by the Department of Agriculture for Northern Ireland. The authors wish to thank Mr Davey McCall for his assistance with the photography, and Dr Chris Selby for his comments.

References

1. Alskieff J and Villemur P (1978) Greffage *in vitro* d'apex sur des plantules décapitieés de pommier (*Malus pumila* Mill). Comptes Rendus Hebd Seance Academie Science, Paris 287: 1115–1118
2. Burger DW (1985) Micrografting: A tool for the plant propagator. Proceedings of the International Plant Propagation Society 34: 244–248
3. Errea P, Felipe A and Herrero M (1994) Graft establishment between compatible and incompatible *Prunus* species. Journal of Experimental Botany 45: 393–401
4. Garner RJ (1979) Chapter 11 The Grafters Handbook, pp 130–222. London: Faber and Faber
5. Gebhardt K and Goldbach R (1988) Establishment, graft union characteristics and growth of *Prunus* micrografts. Physiologia Planatarum 72: 153–159
6. Gersani M (1985) Appearance of new transport capacity in wounded plants. Journal of Experimental Botany 36: 1809–1816
7. Hartmann HT, Kester DE and Davies FT (1990). Chapter 11, Theoretical Aspects of Grafting and Budding. In: Plant Propagation Practices and Principles 5th Edition. Englewood Cliffs: New Jersey Prentice Hall Inc
8. Huang SC and Millikan DF (1980) *In vitro* micrografting of apple shoot tips. Hortscience 15: 741–743
9. Jonard R (1986) Micrografting and its application to tree improvement. In: Bajaj YPS (eds) Biotechnology in Agriculture and Forestry. Trees I, pp 31–48. Heidleberg: Springer
10. Juliano JB (1941) Callus development at the graft union. Phillipine Journal of Science 75: 245–251
11. MacDonald B (1989) Chapters 16–20 Practical Woody Plant Propagation for Nursery Growers, Volume 1, pp 443–591. Portland: Oregon Timber Press
12. McCully ME (1983) Structural aspects of graft development. In: Moore R (ed) Vegetative Compatibility Responses in Plants, pp 71–105. Texas: Baylor University Press
13. Moore R (1984) A model for graft compatibility-incompatibility in higher plants. American Journal of Botany 71: 752–758
14. Monteuuis O and Gautheret R (1986) *In vitro* micrografting of *Sequoiadendron giganteum* meristems. Comptes Rendu Academie Science, Paris 302 Sers 111(6): 223–225
15. Murashige T and Skoog G (1962) A revised medium for the rapid growth and bioassay with tobacco tissue cultures. Physiologia Planatarum 15: 473–479
16. Murashige T, Bitters WP, Rangan TJ, Nauer EM, Roistacher CN and Holliday PB (1972) A technique of shoot apex grafting and its utilisation towards recovering virus free citrus clones. Hortscience 7: 118–119
17. Navarro L, Roistacher CN, and Murashige T (1975) Improvement of shoot tip grafting for virus free clones. Journal for the American Society of Horticultural Science 100: 471–479
18. Navarro L (1988) Application of shoot tip grafting to Woody Species. Acta Horticulturae 227: 43–45
19. Parkinson M and Yeoman MM (1982) Graft formation in cultured explanted internodes. New Phytologist 91: 711–719
20. Parkinson M, O'Neill CM and Dix PJ (1990) Chapter 11 Grafting *In Vitro*. In: Pollard JW and Walker JM (eds) Methods in Molecular Biology vol 6, Plant Cell and Tissue Culture, pp 105–111. New Jersey: Humana Press
21. Pospisilova J, Solarova AJ and Catsky J (1992) Photosynthetic responses to stress during *in vitro* cultivation. Photosynthetica 26: 3–18
22. Rodgers WS and Beakbane AB (1957) Stock and scion relations. Annual Review of Plant Physiology 8: 217–236
23. Simons RK (1986) Graft union characteristics as related to dwarfing in apple (*Malus domestica* Borkh). Acta Horticulturae 160: 57–66
24. Soumelidou K (1994) The anatomy of the developing bud graft union and its relationship to dwarfing in apple. Annals of Botany 74: 605–611
25. Teidemann R (1989) Graft union development and symplastic phloem contact in the heterograft *Cucumis sativus* on *Cucurbita ficiafolia*. Journal of Plant Physiology 134: 427–440
26. Webster AD, Sparks TR and Belcher A (1986) The influence of micropropagation and chemical mutagens on the growth and precocity of Cox's Orange Pippin and Bramley's Seedling Apple. Acta Horticulturae 180: 25–34