

Effect of applied pressure on callus formation and its relevance in grafting

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SUMMARY

Studies were carried out on *Citrus limon* explants *in vitro* to determine the effect of applied pressure on callus formation, cell differentiation and its importance in grafting. Callus was formed earlier in pressure-free explants. The importance of applied pressure in graft formation was indicated by the fusion of callus cells in capped explant experiments. Where callus grew from the edges, a cambium eventually formed within it from the old cambium. The new cambium always curved towards the phloem side, suggesting that a nutrient gradient may be responsible for its location.

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Introduction

Few studies have considered the importance of physical forces and their effects on the processes of callus formation and cell differentiation in plants. According to Lintilhac & Vesecky (1981), the paucity of information may be attributed to the lack of suitable methods of subjecting growing plant tissues to defined mechanical stress. All the methods which have been tried have an inherent problem in that the initially induced stress relaxes over an extended growth period (Yeoman & Brown, 1971). Another major problem is the uncertainty as to the true directionality of the applied stress (Sussex & Clutter, 1968). Other studies which apply mechanical stress on a much larger scale (Quirk & Freese, 1968) are directed at understanding general growth responses, where

RÉSUMÉ

ASANTE, A. K.: *Effet de la pression appliquée sur la formation de cal et sa pertinence en greffage*. Des études se sont déroulées sur les explants de *Citrus limon in vitro* pour déterminer l'effet de la pression appliquée sur la formation de cal, la différenciation de cellule et son importance en greffage. La formation de cal avait lieu plus tôt en explants dépourvus de pression. L'importance de la pression appliquée en formation de la greffe était indiquée par la fusion des cellules de cal dans une expérience d'explant coiffé. Là où le cal se croissait des bords, un cambium se développait à la longue à l'intérieure de l'ancien cambium. Le nouveau cambium courbait toujours vers le côté du phloème, suggérant que le gradient d'une substance nutritive pourrait être responsable pour la détermination de son emplacement.

the applied stresses are mostly supported by woody tissues.

Lintilhac & Vesecky (1981) studied the effects of mechanical stress on cell wall formation in plants and observed that applied mechanical stress appeared to increase the rate of secondary wall deposition in maturing xylem elements. Makino, Kurpda & Shimaji (1983) applied pressure in cultured explants of *Cryptomaria japonica* and observed that the applied pressure suppressed cell proliferation and maintained the integrity of the cambium.

The grafting methods that are used in horticulture bind the graft union with tape to hold scion and rootstock together until the graft partners unite. Studies carried out by Mergen (1954) and Dormling (1963) indicate that pressure

is important for union formation, while recent work by Asante & Barnett (in press) has suggested that pressure at the graft interface may be a key factor in inducing cambial differentiation in callus cells and in uniting the cambia of scion and stock.

This work aimed at studying the effect of applying pressure on callus formation.

Materials and methods

Explants were obtained from pot-grown Rough lemon (*Citrus limon* L.) seedlings grown in a greenhouse at 18 °C. The stem pieces were soaked in 70 per cent v/v ethanol for 60 sec and transferred into 10 per cent v/v sodium hypochlorite containing two to three drops of Tween 20. Sterilization was continued under slight vacuum for 10 min. Subsequently, the specimens were rinsed three times with sterile distilled water in a laminar flow cabinet.

After sterilization, two types of explants were prepared from the stem pieces: (1) The stem pieces were trimmed along the longitudinal edges and further divided along radial planes into smaller blocks of about 2 × 0.3 cm to expose fresh radial and longitudinal surfaces which will be called uncapped explants (Fig. 1A); (2) The stem pieces were cut on longitudinal edges to expose the woody surfaces on both sides of the stem block, and pieces of stem cut from the longitudinal edges were placed on one side of the exposed cut surface. These will be called capped explants (Fig. 1B).

The medium for the explants consisted of

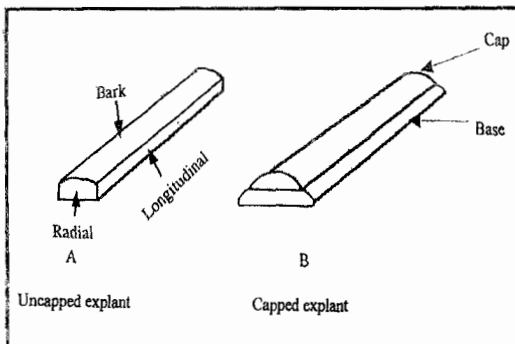


Fig. 1. Types of explants used.

Murashige and Skoog (Murashige & Skoog, 1962) with 0.7 per cent (w/v) Difco agar supplemented with 3 per cent (w/v) sucrose at pH 5.6 and 1.0 mg 2, 4-dichlorophenoxyacetic acid (2,4-D). The hot medium was pipetted into sterile 35-mm culture tubes (8 ml per tube) in which rubber bung had been placed earlier. The cultured tubes were then tilted while the medium was still molten. The explants were placed horizontally, xylem side down, on the rubber bung with the basipetal end of the original stem embedded in the agar slope (Fig. 2). In the culture of uncapped explants, a sterilized stainless steel weighing 100, 50, or 25 g was used to supply pressure while 100 g was used for the capped explants. These were placed on the bark side of the explants to provide the necessary force. Samples of uncapped explants for microscopy were taken after 10, 20, 30, and 40 days and the weight was also measured at each sampling to determine callus development. Capped explants were sampled once at 20 days.

The explants were immersed in Karnovsky's fixative (Karnovsky, 1965) for 4 h and vacuum infiltrated to remove the air from the samples. The specimens were rinsed three times in 0.05 M phosphate buffer at pH 7.0 to remove any unbound fixative, after which they were cut into

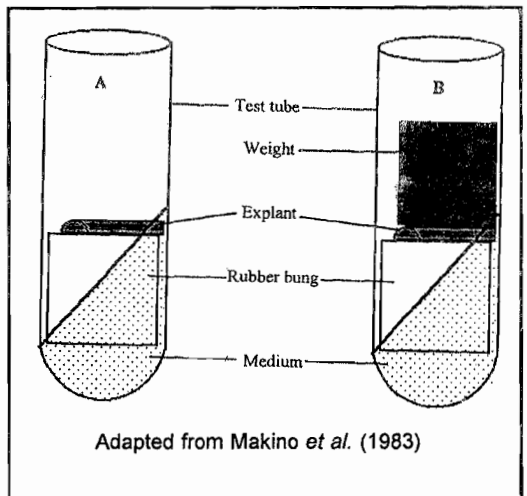


Fig. 2. *In vitro* system of explants; (A) without pressure, (B) with pressure.

smaller pieces about 5 mm in length before post-fixation in 1 per cent w/v aqueous osmium tetroxide for 4 h at room temperature. The specimens were washed three times in distilled water and dehydrated through a graded ethanol series, allowing 30 min in each change. They were then placed in 100 per cent LR White resin (London Resin Company) for 3 days with daily changes of fresh resin. The specimens were finally embedded in fresh LR White resin in large gelatin capsules. Sections 6 μm were cut and stained with safranin (1 per cent w/v in alcohol) at room temperature. The sections were examined and photographed with the Reichert Polyvar II, photomicroscope.

Results

Pressure-free explants

Callus development was well established 10 days after culturing and was more profuse at the radial surfaces than the inner sides of the explants. Callus was produced by the ray cells of xylem and phloem, parenchyma cells in the cortex, and the cambium (Fig. 3). In some instances, callus cells were formed at points where the ray cells dilated close to the cambium layer inside the explant (Fig.

4). It was evident that the outer ray cells were the first to become radially enlarged, protruding beyond other adjacent, immature xylary derivatives. Although some of the immature xylary elements contributed to the formation of callus, the amount they contributed was small in comparison to that produced by the ray cells.

Callus proliferation was vigorous in the cortex, the cambium, and the pith by 20 days, where cambial growth and extension was prominent in the callus around the phloem tissues (Fig. 5). Callus growth increased from 20 days and by 40 days had engulfed almost all sides of the explants,

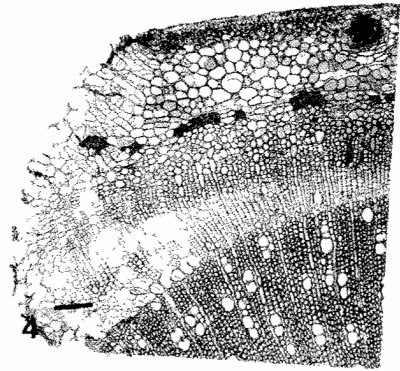


Fig. 4. Transverse section (4 mm) of a 15-day-old pressure-free explant, showing callus development at points where the ray cells appear enlarged in the region of the cambium (arrows). Scale bar = 100 μm .

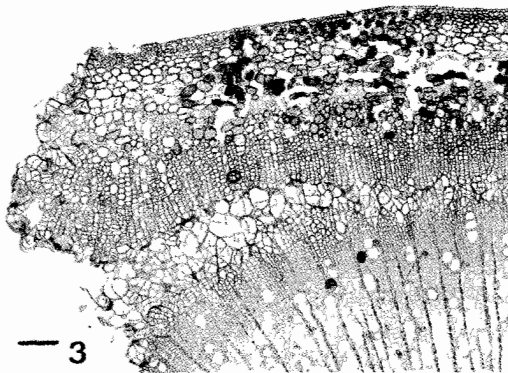


Fig. 3. Transverse section (4 mm) of a 10-day-old pressure-free explant, showing callus formation by the parenchyma cells in the cortex, the cambium, the phloem, and xylem ray cells. Scale bar = 100 μm .

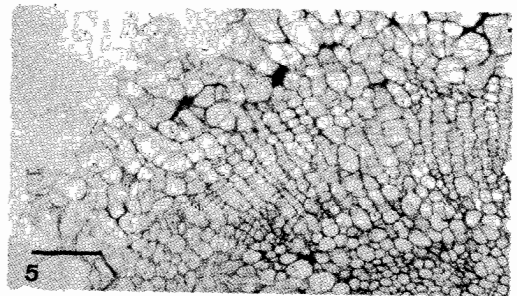


Fig. 5. Transverse section (4 mm) of a 20-day-old pressure-free explant, showing callus proliferation in the cortex of the radial surface. Scale bar = 100 μm .

and cambial cells had developed around the periphery of the phloem zone (Fig. 6).

Callus development increased progressively and was greater in pressure-free explants than in explants under pressure throughout the study.

Callus was formed in the capped explants at 20 days, mostly in the cortical areas of both partners (Fig. 7). The parenchyma cells in the cortex, cambium, xylem, and phloem ray cells all contributed to callus development. However, the main explant and the cap remained separated, since the callus developed could not link up and a clear gap was created between the two.

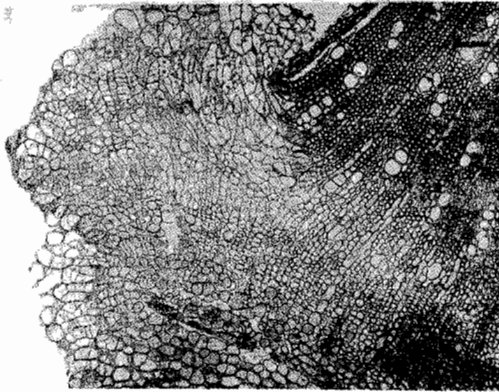


Fig. 6. Transverse section (4 mm) of a 40-day-old pressure-free explant, showing cambium extension around the phloem zone. The callus cells are in rows. Scale bar = 100 μ m.

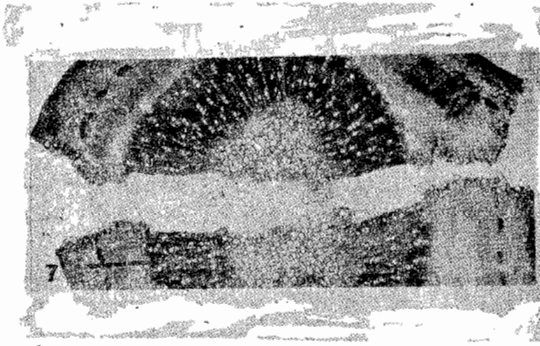


Fig. 7. Transverse section (4 mm) of a 20-day-old pressure-free capped explant. Although callus formation has occurred in the cortical areas, no bond has been established between the partners. Scale bar = 1000 μ m.

Explants under pressure

Under the applied pressure, a layer of young, immature xylem and cambium cells were crushed in some uncapped explants, and these crushed cells remained as a dark zone at the interface, but the more mature xylem cells that had developed secondary walls were unaffected by the pressure (Fig. 8). Callus formation away from the cut edges was hindered by applying pressure; so no callus developed in the explants under 100 and 50 g at 10 days. At 20 days, callus development in explants under 25 g was more pronounced than in any of the other two explants under pressure, and was

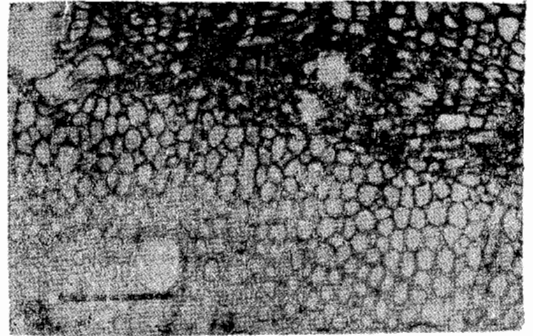


Fig. 8. Transverse section (4 mm) of a 10-day-old explant under pressure (50 mg), showing a layer of crushed young immature xylem and cambium cells which persisted as a dark zone at the interface of the graft. Scale bar = 100 μ m.

limited to the cortex of the radial side mainly by the cambium, xylem, and phloem rays (Fig. 9). Callus growth was observed 30 days at the cut surfaces of all explants under pressure.

By 40 days, callus had covered both the cut surfaces of the explants and cambial extension was very evident. Callus development at 30 and 40 days in explants under 25 g was higher than that in explants under 100 and 50 g.

Callus was formed in the cortex of capped explants cultured under pressure by 20 days, and was observed in both partners (Fig. 10). There was unobstructed mingling of undifferentiated callus cells between the cap and the base explant. The callus union was

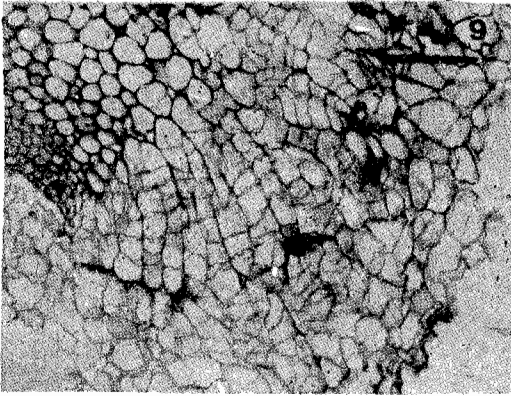


Fig. 9. Transverse section (4 mm) of a 20-day-old explant under pressure (25 g), showing callus development at the radial surface. Callus development has evolved from the parenchyma cells in the cortex, the cambium, the phloem, and xylem rays. Scale bar = 100 μ m.

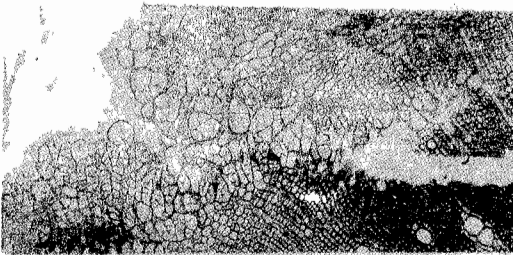


Fig. 10. Transverse section (4 mm) of a 20-day-old capped explant under pressure (100 mg), showing the establishment of callus bond between the partners. The cells at the cortex appear meristematic, showing evidence of division and enlargement. Scale bar = 100 μ m.

complete in the cortex, although the wound edge was not sealed by 20 days. Apart from the cortical area where callus was formed, the rest of the interface was devoid of callus development. Before callusing, the ray cells enlarged and became dilated especially at the points where callus formation was initiated. The callus tissue at this stage was still parenchymatous and entirely meristematic, undergoing random division and enlargement, indicating that callus proliferation was still on-going.

Without pressure, callus was formed along the

cut surfaces and interior interface of explant (Fig. 4); but where pressure was applied, callus was formed with decreasing weight at cut surfaces but absent from the interior interface.

Discussion

The results show that applied pressure prevented callus formation and maintained cells in their original state. When pressure was applied, cell division was immediately halted and callus formation was hindered. However, after a prolonged period in culture, the cells presumably became adapted to the new environment and resumed normal cellular activity.

When cell division began, pressure was generated as a result of enlargement, division, and subsequent callus proliferation, and the effect of applied pressure was nullified. Callus, therefore, developed against the resistive pressure exerted by the applied force. The results show that small applied pressure (25 g) is required initially to prevent the exposed cells from enlarging and dividing; and once physical confinement is restored so that mutual cell pressures are re-established, cambial derivatives enlarge, elongate and divide normally.

More vigorous and profuse callus formation at the radial surfaces than at the inner side of explants under pressure may be due to lack of confinement of the proliferating callus cells at the radial surfaces. The proliferation of the radial cells may also be due to greater availability of oxygen, more rapid release of carbon dioxide, greater availability of nutrients, and more rapid release of volatile inhibitor, or it may be a result of the absorption of products of autolysis by adjacent cells (Yeoman, Naik & Robertson, 1968). The inner part was, however, under direct pressure which prevented an equal rate of callus proliferation as at the free radial sides. The high rate of callus proliferation at the radial surface might also be attributed to its tissues being more exposed than the tissues of the inner side (eg. the ray cells) during the preparation of the explants.

Steward, Marion & Mears (1958) primarily

attributed callus formation at cut surfaces to differences in nutritional gradients caused by an increase in size of the callus mass, resulting in a nutritionally different environment between inner and outer cells of the proliferated callus.

The young, immature cells crushed early in the explants under pressure might be due to a fall in turgor pressure; thus, the cells are unable to withstand the combined pressure and tension from the proliferation of callus masses and the applied pressure.

With the capped explant experiments, it was observed that although callus developed in both explants, the rate of proliferation in the pressure-free explants was higher than in the explants under pressure. Again, this emphasizes the effect of the applied pressure in compressing the cells for some period until a counter pressure was generated in the local cells as they grew.

The applied pressure brought the partners of the capped explants into closer contact, thereby encouraging callus bonding in the region of the proliferating tissue, and diminishing the gap between the partners. Explants cultured without pressure showed a low potential for the proliferating callus to form a union. Similarly, in grafting, pressure is provided by the grafting tape to secure a proper union by holding the partners in place. Neel & Harris (1971) observed that an open wound permits unorganized cell proliferation to form a healing callus, but when this is confined and under pressure, differentiation takes place in accordance with the various stresses governed by depth, orientation, and proximity, emphasizing the need to provide close contact and firm anchorage in grafting. The formation of callus mostly in the ray parenchyma of cambium derivatives and the cortical parenchyma cells agrees with the origin of callus in *Citrus* (Mendel, 1936).

It is of interest to speculate about the stimulus which instigated the preferential path of cambial differentiation around the phloem. Organic nutrients are transported by the phloem, and the

cambium growth and extension around the phloem zone was possibly promoted by the nutrient concentration in the region. In any case, as the cambium growth originated from the old cambium, the effect of hormone as a contributory factor cannot be dismissed readily (Brown, 1964).

Brown & Sax (1962) found that the formation of a new vascular cambium within the callus was initiated from the edges of the original cambium in response to the diffusion of a hormonal stimulant from the cambium. Noel (1968) observed that the position assumed by the new vascular cambium is determined by response to a particular concentration of an unidentified substance, whether nutritional or hormonal, which maintains a concentration gradient across the callus. However, Wilson & Wilson (1961) opposed the idea of hormone diffusing laterally in front of the extending cambium. They suggested that cambium formation is restricted to a particular distance beneath the surface, the distance factor. This is considered to be essentially a physiological gradient stretching from the outer callus inward until at a given position some factor reaches the threshold for cambial initiation.

Brown (1964) observed physiological gradients from the outer exposed callus to the centre. These physiological differences probably result from many physical aspects of the cellular environment, including gas exchange, accumulation of food reserves and waste products, besides mutual pressures and tensions as a result of growth. He stated that the causal factor controlling cambial formation at any given position could still be a hormonal one; the stimulus moves laterally from pre-existing cambium as the regenerating cambium is blocked out in the undifferentiated wound callus.

According to Thair & Steeves (1976), the orientation of vascular cambium is influenced by the directions of flow of nutrients and water in the plant, and can be altered if these lines of flow are changed. However, Colquhoun (1929) inverted patches of bark in *Casuarina paludosa* and examined the secondary xylem that was formed

by the patch; however, no evidence was found that the cambium had reoriented. Vochting (1906) reported that the cambium has an inherent polarity which is independent of its orientation. However, the conclusion remains that the cambium shows a strong resistance to forces which impose a reorientation (Thair & Steeves, 1976).

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