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OPTIMIZATION OF CONDITIONS FOR *IN VITRO* MICROGRAFTING IN RUBBER (*HEVEA BRASILIENSIS*)

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The conditions for successful *in vitro* micrografting such as the nature of cut and type of support, age and size of stock and scion, the physical nature of the medium and effect of antioxidants were standardised for *Hevea brasiliensis*. The micrografting was carried out using zygotic embryo derived plantlets raised *in vitro*. *Ex vitro* micrografting was standardised using polybag grown seedlings as root stocks and scions from glass house grown plants. Maximum success rate was obtained with saddle type of cut with parafilm as the support. Thirty day-old stocks and 21 day-old scions had considerably enhanced the success rate. Scions of length 1.5 cm were ideal for micrografting. Maximum success rate was observed when 60 g/l sucrose was included in the culture medium. Application of the antioxidant ascorbic acid, at a concentration of 100 mg/l to the cut surface of the explants, prevented browning as well as in increased the survival rate. The technique is useful for combining genetically and physiologically comparable rootstocks and scion of elite clones produced by genetic engineering.

Key words: *Hevea brasiliensis*, *In vitro* micrografting, Micropropagation, Zygotic embryos.

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INTRODUCTION

The cultivated clones of *Hevea brasiliensis* (Muell. Arg.) are commonly propagated through budgrafting. Although for a particular clone the scions selected for budgrafting are uniform, the heterogeneity of seedling root stocks lead to stock-scion interactions resulting in considerable variation among the population of a single clone. The need to have many uniform individuals of a selected genotype led to the development of *in vitro* techniques as a means of vegetative propagation. Micropropagation of *Hevea* through shoot tip culture (Asokan *et al.*, 1988; Seneviratne *et al.*, 1993; Seneviratne and Flegman, 1996) and somatic embryogenesis (Etienne *et al.*, 1993a; Carron *et al.*, 1995; Jayasree *et al.*, 1999) have been successful. However, these technologies have not been commercially adopted due to problems in root induction, long time lag and high mortality during hardening and the higher cost compared to conventional propagation by budgrafting.

Micrografting consists of grafting an apex taken from one plant on to another decapitated young plant (Jonard, 1986). This technique has been successfully used in many horticultural plants to develop virus free clones ((Murashige *et al.*, 1972; Navarro, 1988) and to detect graft incompatibilities at an early stage (Herrero, 1951; Mosse, 1962). This method has been proved to be effective as a means of micropropagation of tea (Prakash *et al.*, 1999) and clone rejuvenation of *Hevea* (Perrin *et al.*, 1994) and pine (Travan and David, 1985). Micrografting has been carried out to eliminate viral diseases on different species of lemon tree (Murashige *et al.*, 1972; Roistacher and Kitto, 1977) and fruit trees like cherry (Ozzambak and Schmidt, 1991), kiwi fruit (Ke *et al.*, 1993) and apple (Richardson *et al.*, 1996). In cocoa, where the rooting of *in vitro* grown shoots and regeneration of somatic embryos were problems, this technique was used as a successful alternative for plantlet production (Aquilar *et al.*, 1992). Since 1953,

micrografting has been tried in *Hevea* by grafting cut apices taken from adult plant on to young seedlings to improve propagation of promising clones (Muzik and Cruzada, 1958; Perrin *et al.*, 1994). Similar experiments have also been done on several forest species like *Larix decidue* (Ewald and Kretzschmar, 1996) and *Picea* species (Ponsonby and Mantell, 1993). In most of these attempts, scions raised *in vitro* have been directly transplanted on to stocks raised *in vitro* under sterile conditions. Ke *et al.* (1993) and Luo and Gould (1999) successfully grafted *in vitro* shoots derived from protoplast culture on to mature root stocks of kiwi fruit and transplanted them to the field. The present study was made to understand the different parameters influencing the success of *in vitro* micrografting in *Hevea brasiliensis*. The study has been carried out under *in vitro* as well as *ex vitro* conditions.

MATERIALS AND METHODS

For optimisation of the different parameters, *in vitro* germinated seedlings derived from zygotic embryos were used. After optimising the ideal condition of each parameter, *in vitro* micrografting was tried with *in vitro* grown shoot tip culture derived plants.

Raising seedlings from zygotic embryos *in vitro*

Mature rubber seeds were collected from the *Hevea* clone – RRII 105. After removing the seed coats, the seeds were washed thoroughly in running tap water, surface sterilized with 0.25% mercuric chloride for 5 min and then washed thoroughly with sterile distilled water. The embryos were aseptically dissected out and cultured on MS medium (Murashige and Skoog, 1962) supplemented with growth regulators BA (2.0 mg/l) and thidiazuron (1.0 mg/l). The germinated seedlings were allowed to grow for three weeks before subjecting them to *in vitro* micrografting.

Shoot tip culture derived plants

In vitro shoot tip culture derived plants were produced as described by Asokan *et al.* (1988). Shoot apices of about 2.0 cm length were excised from nursery grown seedlings and surface sterilized with 0.2% mercuric chloride for 3 min. They were then rinsed thoroughly with sterile distilled water and cultured on MS medium with modified major salts (NH_4NO_3-1000 mg/l and KNO_3-1500 mg/l) and growth regulators NAA (1.0 mg/l), BA (2.0 mg/l) and IBA (1.0 mg/l). After culturing for 20 days, they were kept for root induction in hormone free ½ X MS medium. Rooting could be observed after 20 days.

Method of grafting

For micrografting, the method described by Navarro *et al.* (1975) was followed. When the stem axis of the plantlets reached a length of about 2.0 cm the upper 1.0 cm of the tips were excised and used as scion, while the remaining portion was used as root stock. Three types of grafting *viz.*, saddle (/), splice (V) and cleft (T) (Fig. 1) were made in the root stock and scion. The scions were placed in the incisions made in the root stocks with both the cut surfaces in good physical contact (Fig. 2). The stock and scion were held together at the point of graft with sterile parafilm, agar blocks or by mere physical contact.

Optimisation of conditions

To identify the optimum age of the root stock needed for successful grafting, the root stocks derived from 5-50 day old *in vitro* grown seedlings were used. Scions used were glass house grown seedling shoot tips cultured *in vitro* for 7-35 days. Shoot tips taken directly from glass house grown seedlings, for use as scions, were treated as "0" days in culture. *In vitro* grown seedling shoot apices of different length ranging from 0.5 – 3.0 cm were used for standardising the

Fig. 1. Different types of cut used (a) saddle (b) splice (c) cleft

Fig. 2. Micrografting (a) saddle (b) splice & (c) cleft

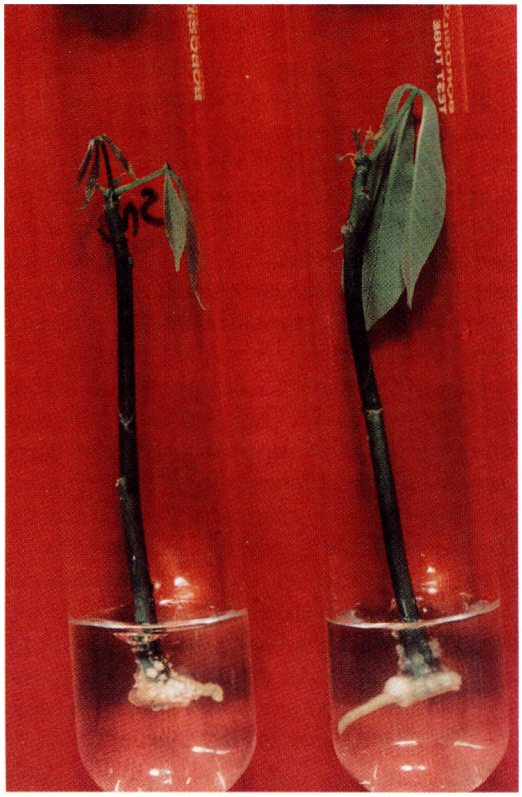

Fig. 3. *In vitro* micrografted plants with apices showing continued growth

Fig. 4. Micrografted plants in polybag

ideal size of the scions. Effect of prior *in vitro* culture of scions was also studied by comparing the *in vitro* cultured shoot tips and those taken directly from glass house. Different types of antioxidants such as ascorbic acid, thiourea and cysteine were applied on the cut surface to study the effect of antioxidants on graft success. The effect of sucrose content in the medium on plant survival was studied by growing the micro grafted plantlets in medium containing different levels of sucrose ranging from 30-80 g/l. For culturing the *in vitro* micrografted plants, both liquid and semisolid media were tried. The pH of all media were adjusted to 5.7. Phytagel, (1.75%) was added as the gelling agent and the medium was autoclaved at 121°C for 15 min. The number of grafts tried for each treatment as 10 and the experiment was repeated thrice. All the cultures were maintained at 26±2°C and at 16 h photoperiod under 20 μEM/2S/I light intensity provided by white cool fluorescent lamp.

The percentage of sprouting apices were determined one month after grafting (Fig. 3). Following the formation of a lignified graft union, which was noticed by continued growth of grafted apex, the micrografts were transferred to polybags (Fig. 4) and hardened by slow reduction of humidity in the glass house. After hardening, the plants were planted in the field.

Ex vitro micrografting

For *ex vitro* micrografting, 15 day old seedlings grown in polybags were used as root stocks. Scions were taken from one month old polybag plants grown in glass house. Saddle, splice and cleft micrografting were done and the grafts were kept in position with budding tape. Grafted plants were kept in glass house with polythene covers to maintain humidity and watered using ½ X MS medium once in a week. Polythene coverings were removed after the grafted apex sprouted and resumed growth.

RESULTS AND DISCUSSION

The type of graft and the nature of support significantly influenced the success of micrograft. Saddle type of cut at the graft gave the most successful micrografts followed by splice method. Cleft type of graft gave the lowest success rate. Saddle type of cut may provide a better surface for the contact of stock and scion providing good cambial connection at the graft union. Of the different supports used, sterile parafilm helped to hold the graft in position better, thus aiding maximum grafting success. For saddle type of cut, maximum success rate was observed with parafilm as the support followed by agar block. For splice and cleft types of cuts, maximum success rate was obtained with parafilm as the support followed by mere physical contact. Maximum graft success rate of 55% was achieved with a combination of 'saddle' type cut and parafilm as the support (Table 1). The results obtained with stock and scions derived from shoot tip derived *in vitro* grown plantlets were also similar to that obtained with zygotic embryo derived seedlings (data not shown). Successful graft intake was also found to be influenced by other factors such as size and age of stock and scion. Maximum successful graft intake was obtained from 30 day old *in vitro* grown seedling derived root stock and 21 day old scions (Table 2). The lowest success rate was obtained when tender matching shoots taken from green house grown seedlings were grafted onto 5 day old *in vitro* grown seedling stocks. The success rate gradually increased with age of stock and scion until they were 30 and 21 days respectively. Beyond that level, the success rate gradually decreased. Similar results supporting the age of the stock and scion for successful micrografting have been reported earlier by Navarro *et al.* (1975).

The sizes of the stock and scion need to be compatible for the scion to fit well into

Table 1. **Influence of type of graft and support (when cultured on media supplemented with 5% sucrose)**

Type of support	Success (%) of type of cut*			Mean
	Saddle	Splice	Cleft	
Parafilm	55.00 (47.90)	48.33 (44.03)	41.66 (40.00)	48.00 (43.90)
Agar block	36.67 (37.23)	32.50 (34.73)	32.50 (34.00)	33.88 (35.56)
Physical contact	35.35 (35.15)	40.83 (39.69)	35.00 (36.00)	36.39 (37.01)
Mean	41.67 (40.08)	40.56 (39.47)	36.39 (37.02)	39.42 (38.42)
CD ($P \leq 0.05$)	-	-	-	4.06

* Mean of 30 grafts x 3 replications; Figures in parentheses indicate angular transformed values

the cut made in the stock leading to successful micrografting (Navarro, 1981). In the present study, the success rate was higher with scions in the range of 1.5 to 2.0 cm length and maximum (85%) being with 2.0 cm long scion. Beyond 2.0 cm scion length, the success rate declined drastically (Table 3).

Both liquid and semi-solid media with different sucrose concentrations were tried for growth of the micrografts. No significant difference was noticed in the growth pattern of micrografts in the two types of media. However, increased sucrose concentration (60 g/l) was found to enhance the success rate and for the subsequent growth of the grafted apex (Table 4). Navarro *et al.* (1975) have reported the influence of carbon source on growth of micrografts. Though the liquid medium could provide increased humidity, the chances for contamination were more.

Table 2. **Effect of age (days in culture) of the stock and scion**

No. of days in culture		No. of grafts	No. of living apices	No. of developed plants	Success (%)
Stock*	Scion*				
5	0	20	6	4	20
10	7	20	9	5	25
20	14	20	13	10	65
30	21	20	18	15	75
40	28	20	12	8	60
50	35	20	12	8	40

* *In vitro* grown seedlings derived from cultured zygotic embryos as stock and tender shoot derived from *ex vitro* grown seedlings as scion with saddle type of cut and parafilm as support.

Table 3. **Effect of length of scion**

Length of scion * (cm)	No. of grafts**	No. of living apices	No. of developed plants	Success (%)
0.5	20	6	4	20
1	20	9	7	45
1.5	20	17	16	80
2	20	18	17	85
2.5	20	15	15	75
3	20	10	9	45

* Scions were seedling shoot tips grown *in vitro*
** Saddle cut and parafilm as support

Another problem encountered was the accumulation of phenols at the cut surface causing tissue discolouration resulting in poor graft union. The use of antioxidants such as ascorbic acid, thiourea and cysteine (100 mg/l) at the cut surface helped in avoiding this. Among the antioxidants used, ascorbic acid at 100 mg/l concentration was found to be most suitable one. The three types of grafts such as splice, saddle and cleft grafts were repeated by incorporating the ideal conditions standardised and the results are given in Table 5. It shows that among the three different types of grafts, both saddle and splice grafts with parafilm support showed success rate of more than 70% and are suitable methods of *in vitro* micrografting for *Hevea brasiliensis*. Since the data expressed in percentage did not follow the normal distribution, the values were subjected to angular transformation prior to analysis of variance. Comparable results were obtained when *in vitro* micrografting

Table 4. **Effect of sucrose concentration and type of medium (for saddle type cut with parafilm support)**

Sucrose concentration (g/l)	Success in semisolid medium (%)	Success in liquid medium (%)	Mean*
30	38.89	37.78	38.33 (38.20)
40	39.26	39.44	39.35 (38.81)
50	59.81	59.45	59.63 (50.59)
60	74.51	71.48	72.73 (58.72)
70	44.26	44.44	44.35 (41.74)
80	39.82	36.11	37.96 (38.00)
CD (P=0.05)	-	-	3.68

* Figures in parentheses indicate angular transformed values

Table 5. **Mean success rate of different types of micrografts under the conditions* standardised**

Type	Mean sucrose rate (%)
Saddle	71.90 (55.91)
Splice	70.90 (57.55)
Cleft	42.36 (40.13)
CD (P≤0.05)	9.80

Figures in parentheses indicate angular transformed values.

* 3 week old seedling derived from cultured zygotic embryos; stem axis 2 cm long; parafilm as support; 100 mg/l ascorbic acid as antioxidant; 6% carbon source.

was done at the optimum conditions identified, using root stocks taken from shoot tip culture derived plantlets also.

Ex vitro micrografting

Three week old seedlings grown in polybags in the glass house were used as root stocks for *ex vitro* micrografting. Scions were either grown *in vitro* or taken from glass house grown plants. For *ex vitro* micrografting budding tape was used to hold the graft in position. This significantly enhanced the rate of grafting success. The results obtained were found to be similar to *in vitro* micrografting (data not shown). Root stocks from three week old seedling performed better. Both younger and older ones usually turned brown and eventually got dried after grafting. Green house grown plants are recommended sources of scions for *ex vitro* grafting since new flushes could be induced at any time of the year. A warm pre-treatment improves the efficiency of graft union. Using *ex vitro* micrografting, saddle and splice grafting methods showed a success rate more than 85 per cent.

Maintenance of grafted plants

Successful *in vitro* micrografts were transferred to polybags and covered with transparent polybags to maintain humidity and kept in glass house. They were hardened by successive reduction of humidity. After hardening, they were transferred to large polybags and planted in the field after one year. *Ex vitro* grafted plants could be transferred to large polybags after one month of grafting and planted in the field after eight months.

It has thus been possible to optimize conditions for *in vitro* micrografting in *Hevea brasiliensis*. This method of propagation has some advantages. Grafting can be done indoors on very young root stocks using *in vitro* multiplied seedlings round the year. This method could also be used *in vitro* for combining different clones possessing desirable characters, propagation of elite materials produced by genetic engineering, identification of clonal and graft incompatibilities and studying physiology of graft union.

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