Journal of Plant Breeding and Crop Science Vol. 3(9), pp. 185-189, 15 September, 2011
Available online http://www.academicjournals.org/JPBCS
ISSN 2006-9758 ©2011 Academic Journals

Full Length Research Paper

In-vitro culture of *Hevea brasiliensis* (rubber tree) embryo

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Accepted 13 July, 2011

In the time past, grafting buds from selected clones on seedlings or plants from seed orchards were used traditionally to propagate rubber trees. Selected seeds were frequently used because their level of resistance to diseases is known. Micro-propagation of rubber tree was the method used in the propagation of *Hevea brasiliensis* in this experiment, which is an approach different from the conventional way of planting rubber and this technique holds good prospect for the future. *In-vitro* culture of *H. brasiliensis* (rubber tree) embryo was carried out using different concentrations of benzylaminopurine (BAP) and 0.01 mg/l concentration of naphthalene acetic acid (NAA) to supplement the murashige and skoog's (MS) medium. In this experiment, shoot and root were obtained from the embryo culture on Murashige and Skoog's (MS) medium supplemented with 0.075 mg/l BAP and 0.001 mg/l NAA. This treatment produced rubber plantlets with well-developed taproots. Using the same concentration of NAA and a BAP concentration slightly higher or lower than 0.075 mg/l, the *H. brasiliensis* embryos produced shoots and lateral roots alone. Embryo cultured in different strength of murashige and skoog's medium (full strength, half strength M/2 and quarter strength M/4) without hormones did not produce taproot. This experiment was unable to achieve mass propagation of rubber plantlets. All the treatment used in the experimentation of *H. brasiliensis* embryo in culture contains 30 g/l sucrose and 7 g/l difco agar.

Key words: *Hevea brasiliensis*, embryo, micro-propagation, *in-vitro* culture, plantlets.

INTRODUCTION

The Brazilian *Hevea brasiliensis*, often called rubber tree is a member of the family Euphorbiceae and the most economically important member of the genus Hevea. It is of a major economic importance because of its timber and latex that can be collected and is the primary source of natural rubber. One of the most well known natural polymers is poly-isoprene or natural rubber. The rubber tree can reach a height of over 44 m (144 ft), para rubber tree (2010).

In the time past, grafting buds from selected clones on seedlings or plants from seed orchards were used traditionally to propagate rubber trees (Asseara et al., 1998). Selected seeds were frequently used because their level of resistance to diseases is known. Since the progress made by switching from multiplication by seed to propagation by budding, the development of new techniques, such as micro propagation, has been awaited (Montoro et al., 2007). For a very long time, selected seeds were preferred to grafts because the resulting trees were more vigorous and displayed better resistance to diseases (Nayanakantha and Seneviratne, 2007). However, from 1940 to 1950, almost all plantations had been established with grafted clones where the trees are comparatively homogeneous, this is because of the

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Table 1. The component of nutrient media used for the culture *Hevea brasiliensis* embryo. The Murashige and Skoog media supplemented with different concentration of BAP and constant NAA concentration was used for all treatments.

Medium	BAP (mg/l)	NAA (mg/l)
A_1	0.025	0.01
A_2	0.05	"
A_3	0.075	"
A_4	0.1	"
A_5	0.15	"

difficulty in obtaining selected seeds and insufficient quantities for developmental programmes (Carron et al., 1989).

There are several reports on Hevea micro-propagation using different explants, mostly derived from seedlings (Jayashree et al., 2000). Thereafter, plantlets with shoots and roots were successfully developed by different investigators (Gunatilleke et al., 1988; Carron et al., 1989; Sompong and Muangkaewngam, 1992; Kyte and Kleyn 1996; Paranjothy and Glandimethi, 1976) induced rooting in plantlets derived from tissue cultures, but were unable to root clonal material, although they regenerated shoots from axillary buds of some clones. Most of the *in vitro* culture work in Hevea is directed towards micro-propagation through shoot tip culture, nodal cultures, somatic embryogenesis and genetic transformation. In Hevea, increased growth and vigour have already been reported for plants regenerated through tissue culture (Carron et al., 1995, 2000).

The experiment was designed to find a suitable growth media that will support the culture of *H. brasiliensis* embryo in growth media at different concentrations of benzylaminopurine (BAP) and a constant concentration of naphthalene acetic acid (NAA) with the Murashige and Skoog media as the basic media of choice. According to Skoog and Miller (1957), the development of plant organs is regulated by the interaction of growth hormones in nutrient medium, which was demonstrated once again in the present experiment.

MATERIALS AND METHODS

The rubber seeds used for the experiment were collected from Ovwor-Olomu in Ughelli South Local Government Area of Delta state Nigeria. All through the period of the experimentation, the rubber seeds used were kept in NACGRAB's gene bank (temperature of -2℃). Eight hundred seeds were used for this experiment. The seeds testae were removed and the cotyledons were washed in running tap water for 2 min. The explants were further washed with tween 20 (two drops) before they were immersed in 70% ethanol for 5 min, thereafter the explants were rinsed with sterile distilled water. Sodium hypo-chloride solution (2% active chlorine) was used for first disinfection (15 min immersion). After washing with sterile distilled water, further disinfection was done using sodium hypo-chloride solution (1% active chlorine) for 10 min. The explants were then rinsed with sterile distilled water for five times. All disinfection process was carried out in the laminar flow hood. Embryos were excised and inoculated in Murashige and Skoog (MS) media of different hormone concentrations as shown in Table 1. After inoculation, cultures were placed in a growth room at 27 ± 1℃ with photoperiod of 16 h light (2000 lux) and 8 h darkness. Four repetitions of the experiment were carried out.

The MS media was supplemented with different concentrations of benzylaminopurine (BAP) ranging from 0.025 to 0.15 mg/l, 0.01 mg/l naphthalene acetic acid (NAA), sucrose 30 g, agar 7 g and inositol 100 mg were used to culture the *H. brasiliensis* embryo. The pH was adjusted to 5.8 before the media was solidified with 7 g/l agar. Five hundred explants were cultured in this experiment, with each treatment having one hundred replicates.

MS media (one-liter) component

Macronutrient (stock 1)

NH_4NO_3 = 33 g, KNO_3 = 38 g, KH_2PO_4 = 8.8 g, $CaCl_2.2H_20$ = 7.4 g and $MgSO_4.7H_2O$ = 3.4 g. Dissolve in distill water then make up to one liter and take 25 ml.

Micronutrient (stock 2)

KI = 0.16 g, $CUSO_4.5H_2O$ = 0.005 g, H_3BO_3 = 1.24 g, $MnSO_4.4H_2O$ = 4.46 g, $Na_2Mo.O_42H_2O$ = 0.05 g and $ZnSO_4.4H_2O$ = 1.72 g and $CoCl_2.6H_2O$ = 0.005 g. Dissolve in distill water then make it up to one liter and take 1 ml.

Vitamins (stock 3)

Nicotinic = 50 mg, *Pyridoxine* = 50 mg, *Thiamine* = 100 mg and *Glycine* = 100 mg. Dissolve in distill water then make it up to one liter and take 10 ml.

EDTA and ferrous sulphate (stock 4)

NA_2 EDTA = 0.07 g and $FeSO_4.7H_2O$ = 0.06 g. Stock 4 are added directly to the media during preparation.

Note: Stocks solutions are stored at 5℃ and the stated volume is collected and adds to the media during preparation.

Table 2. The strength of different MS media used without hormone supplement for the culture. The pH of all media used was adjusted to 5.8 and were solidified with 7 g/l agar.

B_1	MS (full strength that is, undiluted MS media)
B_2	MS/2 (half strength that is, MS media diluted with 50% water v/v)
B_3	MS/4 (quarter strength that is, MS media diluted with 75% water v/v)

Table 3. Development of shoot and root from rubber tree (*Hevea brasiliensis*) after a period 42 days in culture. The component of the media used is shown in Table 1 (treatments A_1 to A_5).

Treatment	Mean value of leaves	Mean value of shoot length (cm)	Number of contamination (%)	Mean value of taproot length (cm)	Mean value of lateral root numbers
A_1	3.08	2.53	0	-	-
A_2	2.00	2.24	5	-	-
A_3	2.50	2.72	10	2.64	3.00
A_4	2.51	2.35	2	-	-
A_5	2.06	2.61	4	-	-

In Table 2, different strength of MS media (MS media diluted with different percentage of water) without hormones supplement was used in this experiment. Three hundred explants were used for this test, with each treatment having one hundred replicates. The cultures were kept in the same temperature of 27 ± 1°C and photoperiod of 16 h light (2000 lux) and 8 h darkness.

RESULTS

In Table 1, after a period of 7 days in culture, embryos in all treatment shown green growth. After 14 days in culture, embryos in treatments A_1, A_2, A_4 and A_5 produced shoot only, while treatment A_3 produced shoot, lateral root and taproot measuring up to an average height of 1.1 cm. After 42 days in culture the rubber plantlets in all A treatments grew up to an average height of 2.49 cm , with only the explants culture on treatment A_3 having taproot. After six weeks in culture, the shoots in treatments A_1, A_2, A_4 and A_5 were transferred to treatment (media) A_3 in order for the shoots to produce root, but they did not produce any taproot, rather they died after 14 days in the new treatment. The level of disinfestations was enough to take care of contamination, as such, very little contamination was recorded.

In this experiment, treatment A_3 with rubber plantlets cultured *in-vitro* possessed well-developed taproots. These were subsequently acclimatized and potted in poly-bags before they were transplanted on the field. The treatment A_3 rubber plantlets that were not acclimatized did not show any sign of further growth *in-vitro*, until they were acclimatized, potted and transplanted on the field. The plantlets that were left *in-vitro* died after a period of three months. Previous work by Paranjothy and Glandimethi (1976) has attempted shoot tip (2 to 3 cm long) culture, derived from aseptically grown seedlings, for the first time. Although these shoots rooted in liquid MS medium, they failed to grow on solid medium. In addition, Enjalric et al. (1982), using shoots derived from 1 to 3 year old greenhouse grown seedlings as explants, developed rooted plantlets.

DISCUSSIONS

Table 3 shows the result of the test carried out on rubber embryo, using different concentrations of BAP while the concentration of NAA was constant. Treatment A_3 produced rubber plantlets with well-developed taproot. These results agree with the work of Asseara et al. (1998) on the development of taproot from axillary buds. Thus, treatment A_3 is the recommended and most appropriate for the *in-vitro* culture of *H. brasiliensis* embryo.

Figure 1a and b show the picture of the rubber plantlets that developed from embryo culture, using treatment A_3.

From Table 2, after a period of 7 days in culture, most embryos showed green growth. After 21 days in culture, majority of the embryos developed into shoot with an average height of 2.55 cm. After a period of 35 days in culture, embryos developed into rubber plantlets with leaves and lateral roots, but there were no taproot in any of the B treatments.

Figures 2a and b shows the picture of the result of rubber embryo culture on MS media without hormone supplements.

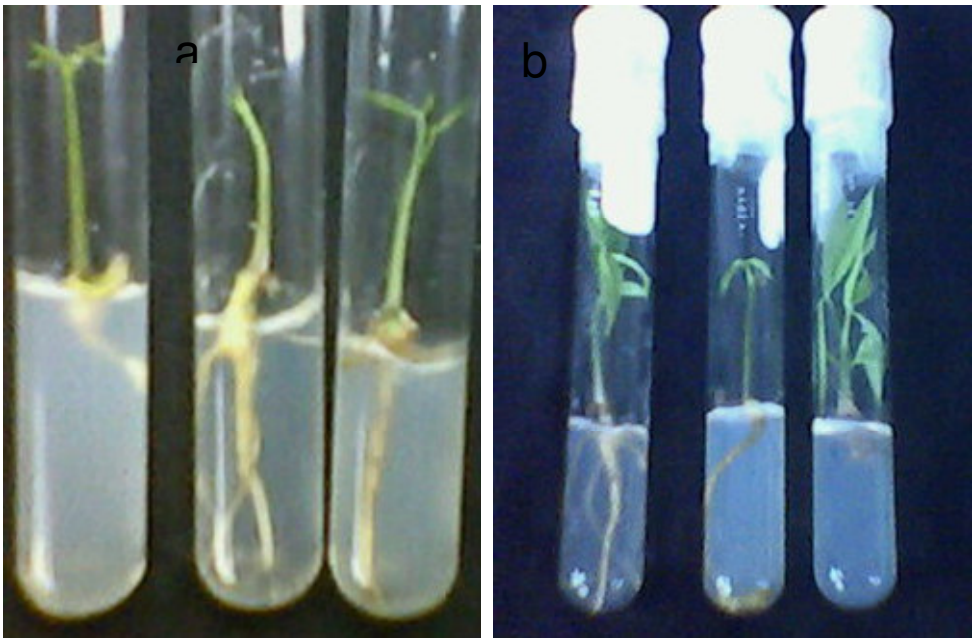

Figure 1. *(a)Havea brasiliensis* after 21 days in culture. (b) *Hevea brasiliensis* after 30 days in culture. (After 21 days, rubber produces taproot and young leaves, leaves become bigger after 30 days in culture).

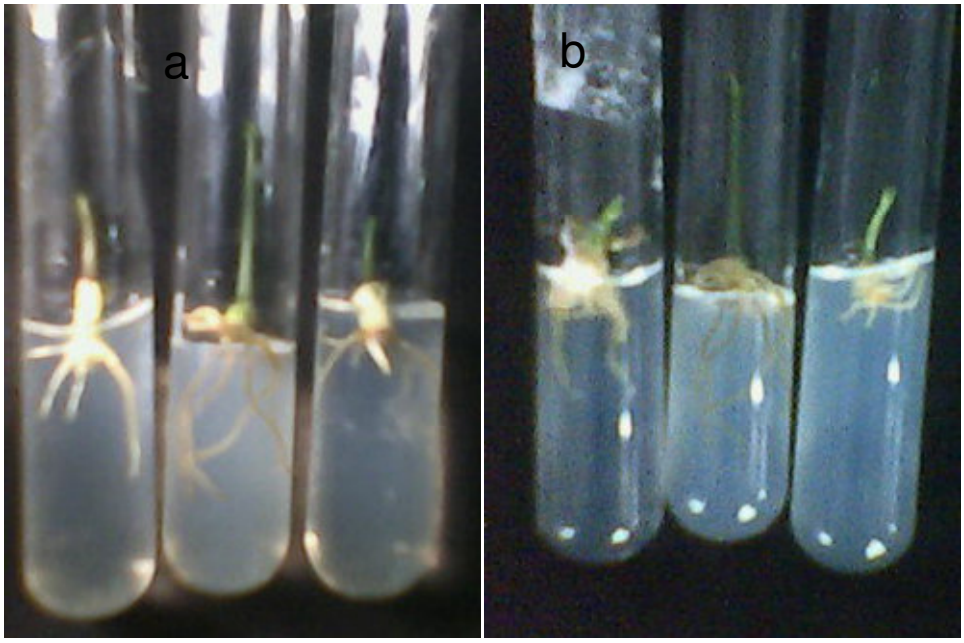

Figure 2. The picture of the result of rubber embryo culture on MS media without hormone supplements. *Hevea brasiliensis* culture on MS media without hormone (Rubber produced lateral roots alone in culture without leaves and taproot after 18 days).

Table 4 shows the result of the test carried out on rubber embryo cultured on different strength of MS media. The result shows that rubber embryo grows in MS media full strength (MS), half strength (MS/2) and quarter

Table 4. Development of shoot and leaves from the rubber embryo, using MS media of different strength without hormone supplement. The component of the media used is shown in Table 2.

Media	Mean value of leaves	Mean value of shoots length (cm)	Number of contamination (%)	Mean value of lateral roots number	Plantlets half life (days)
B_1(MS)	2.07	2.50	2	3.97	45
B_2(MS/2)	2.09	2.55	5	3.46	33
B_3(MS/4)	2.54	2.59	2	3.42	26

strength (MS/4) without hormone supplements. The results agree with the work of Asseara et al (1998) on micro-propagation of rubber tree. The explants cultured on full strength MS media stayed in the in culture for a period of 95 days before they died, while the once cultured in half and quarter strength MS died after 66 and 52 days in culture respectively. These show that the quantity of MS component has effect on the life span of the plantlets in culture.

Conclusion

This experiment was able to find a suitable growth media that produce rubber plantlets with well-developed taproot *in-vitro*. *H. brasiliensis* thrive well in culture that contains 0.075 mg/l BAP and 0.01 mg/l NAA hormone supplements. Mass propagation of *H. brasilienlsis* has not been achieved so far (Asseara et al., 1998). There is need for more research work to be carried out on the possibility of finding a mass propagation media for *H. brasiliensis* in order to produce abundant seedlings of *H. brasiliensis* for planting and the rejuvenation of the falling rubber industries around the world. Due to increase in demand for rubber products, there is need for *in-vitro* technique to be used in the mass propagation of rubber, this techniques produce disease free plantlets which can be carried to different places in large quantity easily. This technique is different from the conventional method of planting rubber and will be able to meet the increase demand of rubber produce.

ACKNOWLEDGEMENTS

The authors thank Mrs. Grace Ekpiwhre, Mr. B.S Erhivwor, Dr Omorovwon and Mr. Ediri Temisere without whom the concept of this research work would not have been conceived. Special thanks to the Director of NACGRAB Mr. W.T Odofin for providing a good atmosphere to carry out this research work.

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