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Growth characteristics of *in vitro* plantlets of *Hevea brasiliensis* obtained from immature embryo culture

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Abstract

Immature pods of *Hevea brasiliensis* were generated through controlled pollination at the Rubber Research Institute of Nigeria for the purpose of embryo rescue. The target was to enhance *Hevea* breeding success in the development of hybrid materials. The pods at ten (10) and twelve (12) weeks old were collected and cultured *in vitro* for 8weeks in 2009 and 2010. Pods were surface sterilized, embryos were isolated and cultured *in vitro* using MS, GB_5) and SH media. At eight weeks in culture, plantlets were taken to the screen house, removed from the vessels and evaluated for growth characteristics: No of leaves, shoot length (cm), No of roots and root length (cm) and were subjected to statistical analysis. The results showed that there was significant variation among the six genotypes in 2009 but no significant difference in 2010 across the parameters determined. Similarly, the three *in vitro* growth media did not differ significantly from one another across the parameters for the two years of the study. With respect to the age of the embryos, 12 weeks old embryos significantly out performed those of the 10 weeks old embryos. Generally, embryos significantly had better performance in 2010 than in 2009. Based on the findings from this study, any of the three growth media can be used successfully for the germination of *Hevea* immature embryos *in vitro*. Also, since the older embryos had better performance than those from the younger embryos, age of immature *Hevea* embryo is a significant factor for consideration to ensure better growth performance *in vitro*.

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Introduction

Hevea brasiliensis belong to the Euphorbiaceae family and it is mostly hybridized by controlled pollination. The major limitation to field success of hand pollination is the abortion of immature pods resulting in low fruit set (Mydin *et al.,* 1990; Omokhafe *et al.,* 2007). The useful tool for the rescue of such embryos or fruits aborted from the mother plant prematurely is the tissue culture technique (Rodrigues-Otubo *et al.,* 2000; Palmer *et al.,* 2002; Burun and Poyrazoglu, 2002 and Niederwieser, 2004).

Successful culture of immature embryos *in vitro* depends on stringent nutritional requirements and constituents of the culture media, which generally differ from species to species (Chopra and Sharma, 1989). According to Sharma *et al.* (1996), successful development of plants from the cultured embryos largely depends on the maturation stage of the embryos.

In tissue culture, once shootlets develop roots and become plantlets, they must be acclimatized before they are planted to the field. Acclimatization of *in vitro* plantlets is an important step in the production system to ensure survival both in the screen house and field (Ng, 2000). The biological and anatomical characteristics of *in vitro* raised-plantlets necessitate that they gradually acclimatize to the green house or field environment (Werbrouch and Debergh, 1994). According to (Chen, 1990), for *Hevea* plantlets to be transplanted from culture vessels to potting mixture and enclosed in a humidity chamber for the purpose of acclimatization, it must develop well-established roots and matured first whorl leaves. This implies that, plantlet's growth vigour is an important factor to be considered in the acclimatization of *in vitro* raised-plantlets either in the screen house or in the field. The objective of this study is to evaluate the growth characteristics of *in vitro* raised-plantlets of *Hevea brasiliensis* prior to acclimatization.

Materials and methods

Location of experiment and source of materials

The study was carried out at the Rubber Research Institute of Nigeria, Iyanomo, Benin City in 2009 and 2010. Immature pods of *Hevea brasiliensis*, which served as the source of the immature embryos (at 10 and 12 weeks after pollination (WAP)), were obtained from an embryo rescue experiment involving six crosses.

Methodology

Seeds were sterilized, embryos were isolated and embryos cultured on three different growth media [Murashige and Skoog - MS (1962), Gamborg *et al.* B_5 - GB_5, (1968) and Schenk and Hildebrandt - SH (1972)]. This gave rise to 6 x 3 factorial combination in a completely randomized design with five (5) cultures per treatment and three replications. Each of these media was supplemented with sucrose (40g/l) and a mixture of NAA (4µM) and BAP (20µM). The pH of each medium was adjusted to 5.8, solidified with 4g/l agar and autoclaved for 20min. at 120°C. An embryo was cultured per culture bottle containing about 20ml of the growth medium. Cultures were maintained in growth room under white fluorescent light for 12h photoperiod at 27 ± 2°C.

Data collection and analysis

At eight weeks in culture, plantlets were taken to the screen house, removed from the vessels and evaluated for growth characteristics: No. of leaves, shoot length (cm), No. of roots and root length (cm). Data were analyzed using Analysis of Variance (ANOVA) according to Gomez and Gomez (1984).Significant means were separated with Least Significant Difference (LSD) at 5% probability level.

Results and discussion

Leaf formation on Hevea plantlets obtained from immature embryos in vitro

The result of the combined analysis for the plantlets obtained from 10 and 12 weeks old embryos in 2009 and 2010 showed that plantlets generated from the 12 weeks old embryos had consistent and significantly higher ($P \leq 0.05$) leaf number relative to those of 10 weeks old at the commencement of acclimatization – 8 weeks old in culture (Tables 1, 2, 3 and 6).

Table 1. Combined analysis for mean number of leaves and roots, shoot and root length obtained through the *in vitro* culture of 10 and 12 weeks old embryos for the six *Hevea* clonal crosses at the commencement of acclimatization in 2009.

	No. of leaves*			Shoot length (cm)*			No. of Roots*			Root length (cm)*		
Crosses	10	12 WAP	Mean	10	12 WAP	Mean	10	12 WAP	Mean	10	12 WAP	Mean
NIG 805 x NIG 804	2	17	9.5bc	0.5	28.2	14.4d	1	31	16.0b	1.0	13.4	7.2a
NIG 805 x NIG 801	0	12	6.0cd	0.0	12.0	6.0e	0	12	6.0c	0.0	6.2	3.1a
NIG 805 x RRIM 600	0	31	15.5a	0.0	40.5	20.3b	0	43	21.5a	0.0	17.6	8.8a
NIG 804 x NIG 801	0	20	10.0b	0.0	27.4	13.7cd	0	32	16.0b	0.0	13.7	6.9a
NIG 804 x RRIM 600	23	12	17.5a	39.3	22.0	30.7a	30	15	22.5a	13.4	5.8	9.6a
NIG 801 x RRIM 600	2	5	3.5d	3.5	12.3	7.9e	5	9	7.0c	3.0	5.3	4.2a
Mean	4.5b	16.2a		7.2b	20.7a		6.0b	23.7a		2.9b	10.3a	

Means followed by the same letter along the column or row are not different significantly at P = 0.05 F-Test.
WAP = Weeks after *pollination*
*: No of embryos with morphological attributes.

This showed that in *Hevea* embryo culture, age of the embryo at the time of culture *in vitro* is of importance such that the older the embryo, the higher its tendency to germinate and grow *in vitro* as was also reported by Fratini and Ruiz (2006). Similarly, among the six crosses in 2009, the plantlets obtained from the embryos of the two crosses NIG 804 x RRIM 600 with a mean leaf value of 10.0 and NIG 805 x RRIM 600 (15.5) differed significantly ($P \leq 0.05$) from the other crosses in terms of number of leaves developed (Table 1). This significance could be attributed to the genetic base of the parents as had earlier been reported by Nunez-Palenius *et al.* (2006) that plant genotype greatly influence plantlet growth *in vitro*.

Table 2. Combined analysis for mean number of leaves and roots, shoot and root length obtained through the *in vitro* culture of 10 and 12 weeks old embryos for the six *Hevea* clonal crosses at the commencement of acclimatization in 2010.

	No. of leaves*			Shoot length (cm)*			No. of Roots*			Root length (cm)*		
Crosses	10	12 WAP	Mean	10	12 WAP	Mean	1 0	12 WAP	Mean	10	12 WAP	Mean
NIG 805 x NIG 804	11	19	15.0a	31.0	52.3	44.7a	30	43	36.5a	22.5	50.8	36.7a
NIG 805 x NIG 801	12	13	12.5a	22.8	45.3	34.1a	28	29	38.5a	19.8	25.2	25.5a
NIG 805 x RRIM 600	10	25	17.5a	20.5	49.3	34.9a	17	46	31.6a	9.0	50.9	30.0a
NIG 804 x NIG 801	5	20	12.5a	13.5	17.5	15.5a	6	22	14.0a	8.5	20.7	14.6a
NIG 804 x RRIM 600	9	16	12.5a	14.5	23.4	19.0a	10	27	18.5a	17.5	17.5	17.5a
NIG 801 x RRIM 600	0	16	8.0a	0.0	48.5	24.3a	0	55	27.5a	0.0	57.8	28.9a
Mean	7.8b	18.2a		17.1b	39.4a		15.2b	37.0a		12.9b	37.2a	

Means followed by the same letter along the column or row are not different significantly at P = 0.01 F-Test.
WAP = Weeks after pollination
*: No of embryos with morphological attributes.

Hevea plantlets cultured with MS growth medium had the highest mean value (57.5) leaf formation in 2009 and 2010 (Table 5).

This did not reflect any significant difference compared to the other two *in vitro* media, except that there was significant variation between the plantlets obtained from 10 and 12 weeks old embryos cultured with the three media across the parameters.

Table 3. Combined analysis for mean number of leaves and roots, shoot and root length obtained through the *in vitro* culture of 10 and 12 WAP across the three growth media at the commencement of acclimatization in 2009 and 2010, respectively.

	No. of leaves*			Shoot length (cm)*			No. of Roots*			Root length (cm)*		
Media	10 WAP	12 WAP	Mean	10 WAP	12 WAP	Mean	10 WAP	12 WAP	Mean	10 WAP	12 WAP	Mean
					2009							
MS	9	31	20.0a	10.8	34.3	22.6a	15	30	22.5a	6.3	13.7	10.0a
SH	5	29	17.0a	12.0	54.0	33.0a	8	55	31.5a	2.4	20.2	11.3a
GB5	10	40	25.0a	18.3	54.0	36.2a	14	57	35.5a	6.0	30.8	18.4a
Mean	8.0b	33.3a		13.7b	47.4a		12.3b	47.3a		4.9b	21.6a	
					2010							
MS	18	57	37.5a	44.5	80.7	62.6a	40	85	62.5a	25.0	102.9	64.0a
SH	16	30	23.0a	28.8	73.0	50.9a	31	61	46.0a	26.3	46.2	36.3a
GB5	12	26	19.0a	29.0	82.6	55.8a	20	74	47.0a	26.0	55.8	40.9a
Mean	15.3b	37.7a		34.1b	78.8a		30.3b	73.3a		25.7b	68.3a	

Means followed by the same letter along the column or row are not different significantly at P = 0.01 F-Test for 2009 & 2010.

WAP = Weeks after pollination, *: No of embryos with morphological attributes

Table 4. Combined analysis of 2009 and 2010 for mean number of leaves and roots, shoot and root length obtained across the six *Hevea* clonal crosses at the commencement of acclimatization.

	No. of leaves*			Shoot length (cm)*			No. of Roots*			Root length (cm)*		
Crosses	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean
NIG 805 x NIG 804	19	30	24.5a	28.7	83.3	56.0a	42	73	57.5a	14.4	73.3	43.9a
NIG 805 x NIG 801	12	25	18.5a	12.0	68.1	40.1a	12	57	34.5a	6.3	45.0	25.6a
NIG 805 x RRIM 600	31	35	33.0a	40.5	69.8	55.2a	43	63	53.0a	17.6	59.9	38.8a
NIG 804 x NIG 801	20	25	22.5a	27.4	31.0	29.2a	32	28	30.0a	13.7	29.2	21.5a
NIG 804 x RRIM 600	35	25	30.0a	59.1	37.9	48.5a	46	37	41.5a	19.2	35.0	27.1a
NIG 801 x RRIM 600	7	16	11.5a	15.8	48.5	32.2a	14	55	34.5a	8.3	38.6	23.5a
Mean	20.7b	26.6b		30.6b	56.4a		31.5b	52.2a		13.2b	46.8a	

Means followed by the same letter along the column or row are not different significantly at P = 0.05 F-Test.

*: No of embryos with morphological attributes.

Shoot formation on Hevea plantlets obtained from immature embryos in vitro

The findings of this study showed that shoot length of *in vitro* plantlets obtained from 12 weeks old embryos in 2009 and 2010 had a significant higher ($P \leq 0.05$) shoot length relative to those of 10 weeks old at the commencement of acclimatization (Tables 1 - 6). A similar trend was maintained in 2009 among the crosses in 2009 with NIG 804 x RRIM 600 differing significantly from the other crosses in shoot length development.

The plantlets of NIG 804 x RRIM 600 had significantly higher shoot length (30.7 cm) compared to those of the other crosses, which range between 6.0 and 20.3 cm (Table 1).

The significant growth of NIG 804 x RRIM 600 over the other crosses could be attributed to the genetic base of the parents as had earlier been reported by Nunez-Palenius *et al.* (2006) that plant genotype greatly influence plantlet growth *in vitro*.

Table 5. Combined analysis of 2009 and 2010 mean number of leaves and roots, shoot and root length obtained from the three growth media at the commencement of acclimatization.

	No. of leaves*			Shoot length (cm)*			No. of Roots*			Root length (cm)*		
Media	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean
MS	40	75	57.5a	45.1	125.2	85.2a	45	125	85.9a	20.0	127.9	74.0a
SH	34	45	39.5a	66.0	101.8	83.9a	63	92	77.5a	22.6	72.5	47.6a
GB5	50	38	44.0a	72.3	111 .6	92.0a	71	94	82.5a	36.8	81.8	59.3a
Mean	41.3a	52.7a		61.1b	112.9a		59.7b	103.7a		26.5b	94.1a	

Means followed by the same letter along the column or row are not different significantly at P = 0.01 F-Test. *: No of embryos with morphological attributes.

Root number and root length of Hevea plantlets obtained from immature embryos in vitro

In terms of root development and growth, the results of the combined analysis for the plantlets obtained from 10 and 12 weeks old embryos in 2009 and 2010 showed that, plantlets generated from the 12 weeks old embryos had a higher ($P \leq 0.05$) number of roots and root length, which differed significantly from those of 10 weeks old embryos at 8 weeks old in culture (Tables 1 - 6). As observed in other parameters, age of an embryo at the time of culture is vital to its growth and development *in vitro*.

Table 6. Combined analysis of 2009 and 2010 mean number of leaves and roots, shoot and root length obtained for the embryo ages at the commencement of acclimatization.

	No. of leaves*			Shoot length (cm)*			No. of Roots*			Root length (cm)*		
Embryo age	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean	2009	2010	Mean
10 wks. old	24	46	35.0b	41.1	102.3	71.7b	37	91	64.0b	14.7	77.3	46.0b
12 wks. old	100	113	106.5a	142.3	236.3	189.3a	142	220	181.0a	64.7	204.9	134.8a
Mean	62.0a	79.5a		91.7b	169.3a		89.5b	155.5a		39.7b	141.1a	

Means followed by the same letter along the column or row are not different significantly at P = 0.05 F-Test. *: No of embryos with morphological attributes.

Two crosses NIG 804 x RRIM 600 and NIG 805 x RRIM 600 developed significant ($P \leq 0.05$) higher number of roots 22.5 and 21.5, respectively compared to those of the other genotypes, which ranged between 6 and 16 in 2009 (Table 1). This reflects the fact that every living organism expresses itself genetically under normal conditions. The significant root development of these two genotypes over the other crosses could be attributed to the genetic base of the parents as reported by Nunez-Palenius *et al.* (2006) that plant genotype greatly influence plantlet growth *in vitro*.

Conclusion

The *in vitro* growth characteristics of the plantlets from the six genotypes investigated showed that any of the three tested *in vitro* growth media can be used successfully for the *in vitro* culture of *Hevea* immature embryos.

The genetic background of the embryos and the age of the embryo at the time of culture had significant impact on the growth and development of the *Hevea in vitro* plantlets at this stage. Generally, the *in vitro* plantlets developed adequate features to ensure their survival both in the screen house and field.

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