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Full Length Research Paper

Studies on *Hollarrhena floribunda (*G. Don) Durand & Schinz: Germination and seedling growth, and preliminary investigations on callus induction and plant regeneration

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Seed germination, seedling growth and *in vitro* regeneration studies were conducted on *H. floribunda* (Apocynaceae), a potential reforestation candidate. The seeds germinated readily with 96.0 ± 2.5% germination in 10 days. The seedlings exhibited a slow growth rate, attaining a shoot height of 35.5 ± 4.46 cm and a leaf area of 23.86 ± 3.89 cm^2 in 12 weeks. Murashige and Skoog (MS) medium supplemented with various concentrations of 2, 4-dichlorophenoxyacetic acid, (2, 4-D) alone or combined with Benzyladenine induced callus formation in leaf, internode and node explants. Massive callus production was generally observed with explants cultured on MS media supplemented with 4.5 µM 2, 4-D alone or combined with 2.2 µM BA. The highest frequency of shoot regeneration was induced by a combination of 4.5 µM 2, 4-D and 2.2 µM BA; no complete plantlets were however produced.

Key words: Callus, germination, *H. floribunda,* plant regeneration, seedling growth.

INTRODUCTION

Holarrhena floribunda (G. Don) Durand and Schinz, known as false rubber belongs to the family Apocynaceae. It is a small to medium size tree (10 to 15 m) depending on the ecological zones (Tamboura et al., 2005). It is a highly valued multipurpose tree. The shinning leaves that is mostly ovate to lancelolate and the white fragrant flowers in almost umbel-like inflorescences make *H. floribunda* an attractive ornamental. The plant is of ethnomedicinal importance: the stem, bark and leaves are widely used in West African countries in the treatment of malaria (Fortie et al., 2006); the leaves are also used in the treatment of reproductive disorders in livestock in Burkina Faso (Tamboura et al., 1998). In addition, the white wood of *H. floribunda* is used for carvings and the floss obtained from the seeds serve for stuffing pillows. Today, there is a growing concern throughout the world about the uncontrolled and depletion of the earth's natural resources especially those affecting plant diversity of tropical forests (Jayachandra et al., 2009). One of several reasons is because plant resources continue to play a central role in the livelihood of the rural poor in Africa. First, we humans use many plant products directly as food. Okafor (1993) reported that 171 indigenous woody plants are of nutritional importance within the forest zone of Nigeria. Second, wood is a source of fuel for the African poor who have no access or are unable to afford the price of coal, kerosene or gas. It is estimated that over 90% of the people of Africa depend on either firewood or charcoal for cooking and heating (Temu, 2002), a trend that may not be reversed in many decades to come (UNDP, 2002). Third, medicinal plants are said to form the largest grouping of plant with about 30,000 species used for medicine world wide and nearly a third of them are trees (Schmelzer, 2008). If many of the forest trees in Africa are not to go into extinction and if the socio-economic condition of the rural poor in Africa

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is not to be worse off and if the consequences of the disappearance of forest *viz* accelerated soil erosion,declining soil productivity, flooding etc. are to be minimized there is an urgent need for reforestation, throughout African countries. This need cannot be satisfied by the conventional methods of propagation; rather micropropagation which allows for the production of large number of plants will be required.

The application of micropropagation is well documented in the literature with respect to production of ornamentals (Lu et al., 1990; Ahmed et al., 2002), food crops (Compton and Gray, 1993; Gopal et al., 1998; Shirin et al., 2007) and forest trees (Gupta et al., 1980, Pandey et al., 2006, Agrawal and Sandar, 2007).

A lot of phytochemical and pharmacological studies have been reported on *H. floribunda* (Fortie et al., 2006, Badmus et al., 2010), a highly valued multipurpose tree but there is no information on its early seedling growth or on its micropropagation in the literature to our knowledge. This paper reports the seed germination and seedling growth of *H. floribunda* and the preliminary experiments carried out on the development of protocol for callus induction and multiple shoot production from its various explants.

MATERIALS AND METHODS

Dry brown fruits of *H. floribunda* were plucked from the tree stands in the Reforestation Project Site of Obafemi Awolowo University and the seeds were extracted from the capsules, washed with tap water and distilled water in which they were also soaked for one hour for imbibitions. The seeds were then sown on moist filter paper in Petri dishes and incubated at 25±2°C in the dark. Four replicates of 25 seeds were sown and the germination experiment was repeated thrice. Counts of germinated seeds were made in an interval of 2 days for a period of 10 days. Visible breaking of the seed coat by the radicle was taken as criterion for germination.

Seedling growth and measurement

Humus top soil obtained from the forest patch in front of the Department of Botany, Obafemi Awolowo University Ile-Ife, and was distributed into clean plastic bowls 25 cm in diameter and 10 cm in depth. Each bowl had four holes (each with diameter of about 0.5 cm) bored at its bottom to allow for drainage. Two-week old seedlings of *H. floribunda* each with radicle length of about 2 cm were transplanted into the bowls (5 seedlings per bowl) and were placed under shade for one week to avoid wilting after which they were placed in an open space to receive full sunlight. Each bowl was supplied with 200 ml of distilled water each morning throughout the period of growth studies. Beginning from 4 weeks after the seeds were sown (that is, 2 weeks after transfer to full light), the following growth measurements were taken fortnightly for 8 weeks.

Shoot height

The shoot height for each plant was measured as the distance between the base of the shoot at the soil level and the tip of the terminal bud of the plant using a thread and meter rule. A total of 30 plants were measured, and the mean value and standard error calculated.

Leaf area

The leaf area of every leaf for each plant was measured by placing each leaf on a 15 × 10 cm graph paper glued to a glass plate. After marking the leaf outline with a marker, the area was determined by counting the squares to the nearest 1 mm^2. The average leaf area per plant and standard error was then calculated based on 30 plants.

Leaf number

The total number of leaves for each of 30 plants was counted and the average number per plant and standard error calculated.

Callus induction and shoot regeneration

Explants of leaf, internode and node of seedlings (4 to 6 weeks old) of *H. floribunda* were used for callus induction and regeneration experiments. The explants were first washed under running tap water and sterilized with 70% ethanol for 1 min and then 10% commercial bleach (Jik) for 10 to 15 min after which they were rinsed in three changes of distilled water before inoculation. Leaf explants, wounded on all sides were cut into segments about 1 cm^2 after which they were inoculated on the medium with abaxial surface facing up. Stem explants were cut into approximately 1 cm segments and placed horizontally on the surface of the medium while nodal explants were inoculated vertically on the culture medium.

Callus induction was carried out on modified Murashige and Skoog's (1962) basal medium supplemented with 2.25, 4.5 or 6.75 µM 2, 4-Dichlorophenoxy acetic acid (2,4-D) or each of these concentration of 2,4-D separately combined with 2.2 or 4.4µM Benzyladenine. Callus proliferation or shoot regeneration was induced by sub-culturing callus on 2.2 or 4.4µM Benzyladenine. Twenty five explants were used for each medium treatment and the experiment was repeated three times. The cultures were incubated in the dark in a Gallenkhamp incubator, H270 at 25 ± 2°C and the responses of the cultures to callus-inducing media were scored rather than accurately measured to aid a more rapid screening options and to use the findings as input to subsequent studies (Cousins and Saenger, 2002). Explants that produced more than two shoots were considered as multiple shoots. Frequencies of responses of cultures with respect to callus induction or multiple shoot formation were determined with standard errors.

RESULTS

Germination and seedling growth

The percentage germination of the seeds of *H. floribunda* over time is shown in Figure 1. It was observed that 2.5% of the seeds germinated by the second day of incubation and the germination progressed with time reaching 77.5% by day 8 and 96% by day 10.

The seedling transferred to soil increased in growth with time with respect to shoot height, number of leaves and leaf area. The mean shoot height increased significantly from 4.9 ± 0.88cm in week 4 to 35.5 ± 4.46 cm in week 12 (Figure 2) while the mean leaf area increased

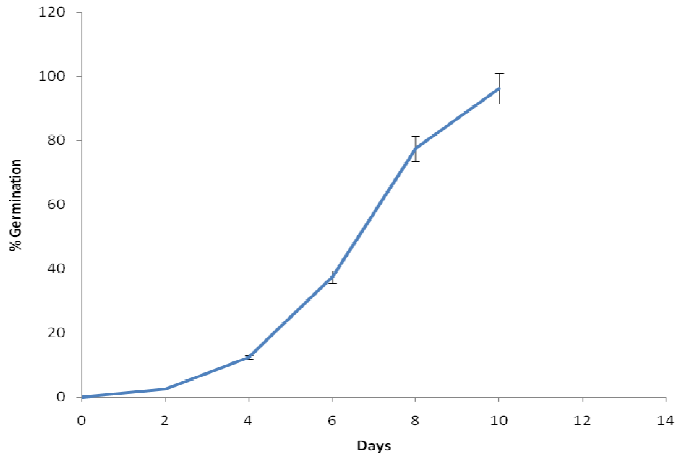

Figure 1. Graph showing germination percent of *H. floribunda* seeds in the dark at 25 ± 2°C

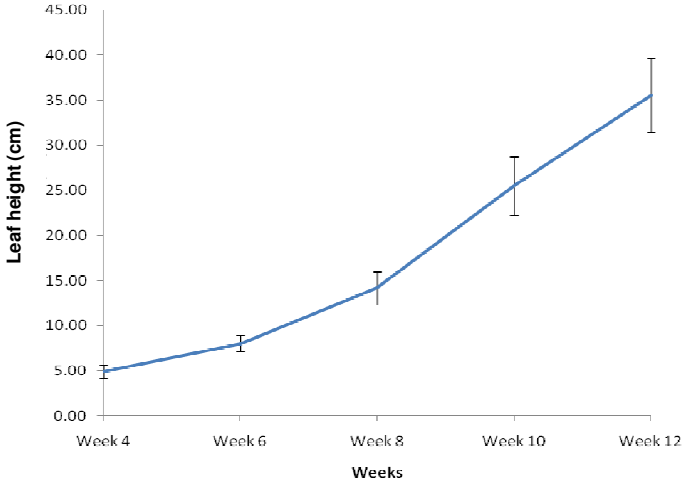

Figure 2. Graph showing the mean shoot height (cm) of *H. floribunda* from 4 weeks after sowing to 12 weeks after sowing.

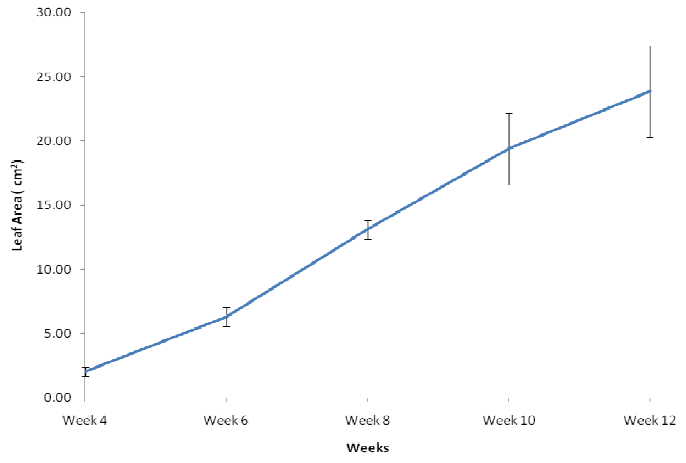

Figure 3. Graph showing mean leaf area of *H. floribunda* from 4 weeks after sowing to 12 weeks after sowing.

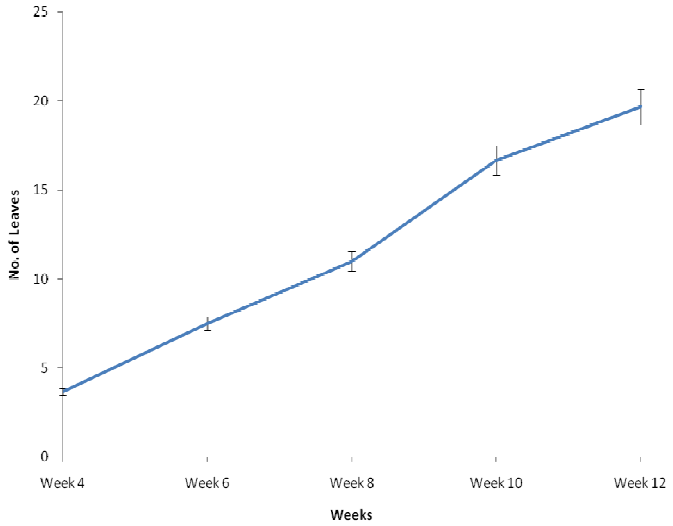

Figure 4. Graph showing the number of leaves of *H. floribunda* from 4 weeks after sowing to 12 weeks after sowing.

from 2.06 ± 0.40 cm^2 in week 4 to 23.86 ± 3.89 cm^2 in week 12 (Figure 3). The leaves which emerged in pairs and oppositely arranged showed significant increase in number from 4 ± 0.04 cm in week 4 to 20 ± 0.25 cm in week 12 (Figure 4).

Callus induction and regeneration

The internode segments inoculated on MS basal medium expanded in length and breadth throughout the period of incubation without callus formation from the cut ends. Those segments on MS medium supplemented with plant growth regulators were observed to have callus initiation from the cut ends between 11 to 24 days; the earliest callus initiation was obtained in 6.75 µM 2, 4-D although it turned brown within 3 weeks. Massive production of calli were found with MS media containing 4.5 µM 2, 4-D alone and/or combined with 2.2 µM BA. The calli in both cases were hard, nodular and pale green (Figures 5a and b) and when sub-cultured on MS to which 2.2 or 4.4 µM BA had been added, a little more cell proliferation was observed in the callus which remained hard and nodular. No shoot buds developed from them after 8 weeks of sub-culture.

The nodal explants cultured on MS media alone produced single axillary buds that grew very much like a seedling reaching an average height of 4.2 ± 0.85 cm after 5 weeks of culture (Figure 6a). When the two single nodal explants excised from the shoot were transferred to MS media containing 2.25 µM 2, 4-D for rooting there was little or no further growth of the shoots and no roots developed until the shoots became necrotic and died after 6 weeks of sub-culture. In the case of nodal cuttings inserted into MS media supplemented with 2,4-D alone, the general trend was production of rather hard and brown

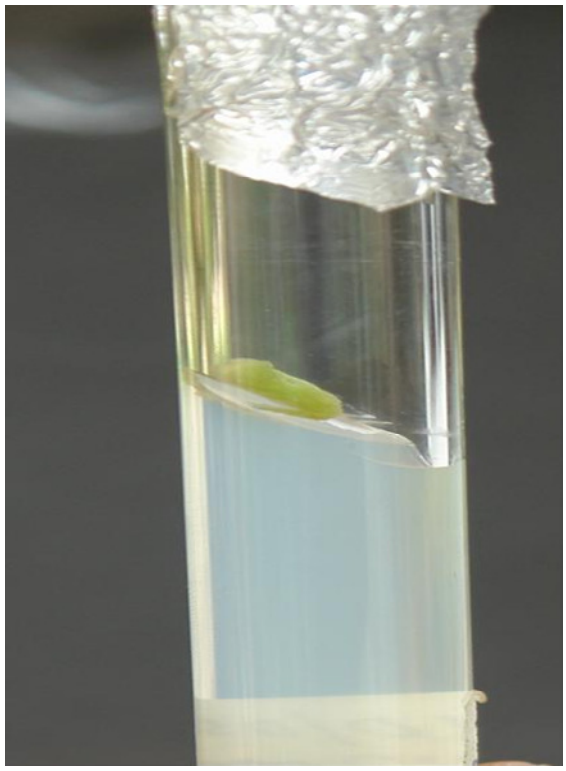

Figure 5a. Callus formation in internode explant of *H. floribunda* on MS medium supplemented with 4.5 µM 2, 4-D after 4 weeks of culture.

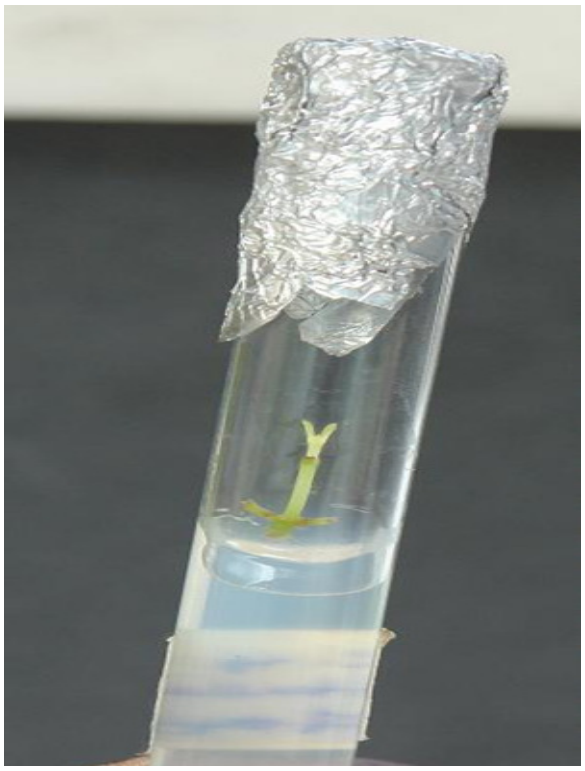

Figure 6a. Shoot produced from nodal explant of *H. floribunda* cultured on hormone-free MS medium for 5 weeks.

Figure 5b. Callus formation in internode explant of *H. floribunda* on MS medium supplemented with 4.5µM 2, 4-D + 2.2 µM BA after 4 weeks of culture.

Figure 6b. Callus induction from nodal explant of *H. floribunda* cultured for 3 weeks on MS medium containing 4.5 µM 2, 4-D.

Figure 6c. Callus proliferation of nodal explant - derived callus of *H. floribunda* subcultured for 2 weeks on MS medium supplemented with 4.5 µM 2, 4-D.

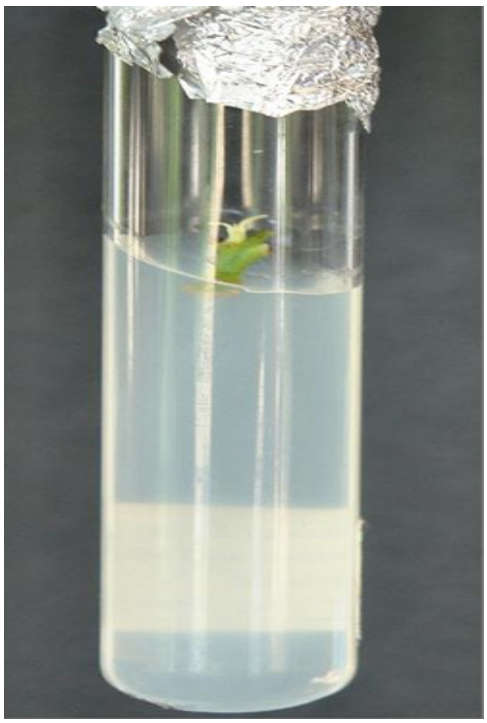

Figure 6d. Callus and shoot formation in nodal explant of *H. floribunda* cultured on MS medium containing 4.5 µM 2, 4-D and 2.2 µM BA for 3 weeks.

callus from the basal cut ends and a cream coloured soft callus from the upper cut ends (Figure 6b). It was observed that the nodal explants cultured on MS media containing 4.5 µM 2, 4-D gave the highest percentage response, 81%. Clumps of the basal callus sub-cultured on the same media produced fast growing callus within two weeks of culture. The calli formed were friable, nodular and brownish in colour (Figure 6c).

The nodal explants grown on MS media to which different concentrations of 2, 4-D were separately combined with BA generated callus from which 2/3 axillary shoots were produced (Figure 6d). The percentage response for each combination is shown in Table 1. Media supplemented with 4.5 µM 2, 4-D and 2.2 µM BA produced the highest frequency (58%) of shoot regeneration. When the shoots were transferred to hormone-free media or MS media with low concentration of 2, 4-D (2.25 µM), no roots were formed after 6 weeks of subculture.

The leaf explants cultured on MS medium that lacked plant growth regulator expanded to half again its original area within the first 2 weeks. Callus initiation with subsequent little cell proliferation was observed after 58 days of culture. The callus was soft, white, and smooth. Various concentrations of 2, 4-D added to MS media induced callus initiation from the leaf explants between 38 to 45 days (Table 1). The callus induction frequency was highest (41%) in MS medium containing 4.5 µM 2, 4-D and the callus was smooth, soft and white (Figure 7). Clumps of the calli sub-cultured on media containing either 2.2 or 4.4 µM BA did not produce shoots.

The leaf explants inoculated on MS medium supplemented with combination of 2,4-D and BA in general became wrinkled initially after 2 weeks of culture and subsequently produced callus which was hard, nodular and green in colour (Figure 7b). When the nodular sections were sub-cultured on MS media containing either 2.2 or 4.4 µM BA, no development of buds were observed; the nodules became more pronounced but turned brown after 6 weeks and died.

DISCUSSION

The high percentage germination observed for *H. floribunda* seeds shows that the seeds are not dormant. The seeds may however have physiological dormancy (www.kew.org). *H. floribunda* occurs in deciduous forest, open localities in dense forest, woodland and savanna and the germination capacity of the seeds is likely dependent upon the seasonal patterns of the species native habitat. The seeds used in our study were freshly harvested and obtained from tree stands in Obafemi Awolowo University campus Ile-Ife, Nigeria (7.55°N, 4.5°E) with 2 prominent seasons (rainy and dry) and an annual temperature range of 27 to 34°C. Those seeds stored at 5 ± 2°C showed a percentage germination of less than 30% within 1 month of storage and less than

Table 1. Effect of various combinations of 2,4-dichlorophenoxy acetic acid (2, 4-D) alone or separately combined with Benzyl adenine (BA) on callus induction or shoot regeneration**.**

Type of explant	Growth regulator		Time of initiation of callus (days)	% Explant with callus	Degree of callus formation	Shooting response (%)
	2, 4-D (µM)	**BA** (µM)				
Stem internodes	0	0	0	-	-	
	2.25	0	24	40 ± 2.5	+	
	4.5	0	16	71 ± 4.5	+++	
	6.75	0	11	30 ± 1.5	++	
	0	2.2	18	42 ± 2.0	+	-
	2.25	2.2	24	38 ± 1.8	++	-
	4.5	2.2	10	81 ± 4.2	+++	-
	6.75	2.2	14	12 ± 2.3	++	-
	0	4.4	20	58 ± 3.9	++	-
	2.25	4.4	17	47 ± 2.6	++	-
	4.5	4.4	18	75 ± 3.8	++	-
	6.75	4.4	19	26 ± 1.53	+	-
Nodal explant	0	0	0	-	(S)	60 ± 4.8
	2.25	0	12	60 ± 2.8	++	
	4.5	0	18	81 ± 3.6	+++	
	6.75	0	36	32 ± 1.5	+	
	0	2.2	15	51 ± 2.5	+	
	2.25	2.2	19	56 ± 2.7	++	42 ± 4.2
	4.5	2.2	16	61 ± 3.1	+++	58 ± 5.6
	6.75	2.2	22	28 ± 2.6	+	-
	0	4.4	21	56 ± 1.9	++	-
	2.25	4.4	19	44 ± 2.9	++	20 ± 2.1
	4.5	4.4	21	55 ± 3.1	++	40 ± 3.8
	6.75	4.4	36	32 ± 1.2	+	-
Leaf explant	0	0	58	5	+	
	2.25	0	40	26 ± 1.0	++	
	4.5	0	38	41 ± 2.1	++	
	6.75	0	45	28 ± 2.3	+	
	0	2.2	56	37 ± 1.5	+	
	2.25	2.2	47	52 ± 4.1	++	
	4.5	2.2	42	64 ± 3.5	+++	
	6.75	2.2	52	12 ± 1.2	+	
	0	4.4	49	36 ± 2.1	++	
	2.25	4.4	42	48 ± 2.3	+	
	4.5	4.4	40	50 ± 1.8	+	
	6.75	4.4	36	20 ± 1.5	+	

*The values represent the means (±SE) of three independent experiments, each using 25 explants. + = Low callus formation ; +++ = Massive callus formation, ++ = Moderate callus formation, S = Single Shoot.

20% within 2 months of storage (Personal Observation). The observation of decrease in the percentage germination of the seeds in storage may be due to decrease in viability or development of secondary dormancy. The seedling growth rate with respect to shoot height from week 4 to 6 was found to be slow although it reached a plantable size seedling, shoot height 25 cm by week 10. The food reserve of the seeds of *H. floribunda* is quite small and this may account for the initial slow growth rate of the seedling. A significant increase in the shoot height was, however, observed from week 8 onwards. This is due to the significant increase in the number of leaves and leaf area during the period leading to increase in photosynthetic activity and hence increased food production, a prerequisite for growth.

The two methods of plant regeneration widely used in

Figure 7a. Callus induction from leaf explant of *H. floribunda* cultured on MS medium alone for 5 weeks.

Figure 7b. Callus induction from leaf explant of *H. floribunda* cultured on MS medium containing 4.5µM 2, 4-D and 2.2µM BA after 2 weeks.

rapid and large scale micropropagation or plant transformation studies are organogenesis and somatic embryogenesis. In either case, callus formation may be involved and hence the pathways are described as indirect organogenesis or indirect embryogenesis respectively. In our study, callus formation was found to be induceable from leaf, internode and node explants of the seedlings of *H. floribunda*. MS medium fortified with 4.5 µM 2,4-D alone or in combination with 2.2 µM kinetin highly promoted callus induction at high frequencies of 71 and 81%, respectively from internode segments. Martin (2000) reported callus induction from internode explants of *Holostemma ada-kodien* cultured on MS medium to which 0.5 to 2.0 mg/L NAA/IAA alone or in combination with 0.5 to 2.0 mg/L kinetin/BAP was added. According to Da Silva et al. (2005) 2,4-D is the main synthetic auxin used for induction of callogenesis because it has great capacity to efficiently stimulate cell division of tissues in several plants. NAA and 2, 4-D are commonly used with BA for callus induction in various plant systems (Dhar and Josh, 2005; Agrawal and Sadar, 2007; Abbasin et al., 2010).

Callus formation at the cut surface of the leaves was observed in hormone free media after 58 days of culture. Callus formation without exogenous plant growth regulator, a consequence of wound reaction (Khal, 1983) has been reported by several workers (Martin, 2002; Handro and Floh, 2001). Inclusions of 2, 4-D to the basal MS medium brought about earlier initiation of callus from the leaf explants while 2,4-D combined with BA induced callus production that was nodular. The nodular callus however did not produce shoot when further sub-cultured on either hormone-free or MS basal media supplemented with BA. This might be because the concentration of the growth regulators used were not optional, otherwise nodules are spherical or globular morphogenic manifestation which have potential to develop into embryoids or ordinary shoots (Ehsanpour, 2002). Xie and Hong (2001) obtained the highest percentage of adventitious shoots from the nodules of *Acacia magnum* on medium containing 0.5 µM TDZ (thidiazuron). *In vitro* regeneration of plantlets have been reported in leaf explants of several species (Morini et al., 2001; Feyissa et al., 2005)

Nodal explants of *H. floribunda* cultured on MS basal media alone produced single axillary shoot that developed to a height of 4 cm within 5 weeks of culture. Such single nodes when sub-cultured can in turn grow into similar seedling like shoots, a process which can be repeated for further multiplication. Nodal explants cultured on media to which a combination of 2, 4-D and BA had been added generated both callus and shoots. Micropropagation protocols based on the tendency of excised nodes to form single or multiple axillary shoots have been utilized to clone a number of plant species (Sharma and Chindall, 1992; Al-Bahrany and Al-Khayari, 2003). More recently, Elavazhagan and Arunachalam (2010) induced callus and multiple shoots from nodal

explants of *Memelon edule* grown under light and dark conditions on MS half-strength medium supplemented with IAA, 2,4-D and kinetin combinations. Similarly, Sajeevan et al. (2011) induced multiple shoots from nodal explants of *Morus alba* L. variety VI with 1 mg/L BAP, 0.1 mg/L TPZ and 0.25 mg/L NAA.

In conclusion, the results reported here have paved the way for a rapid and large scale micropropagation of this highly valuable tree species in addition to providing information on the germination of the seeds and the seedling growth rate. Further investigations are however required for optimization of the protocol for multiple shoot production and plantlet regeneration.

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