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Vegetative Propagation and Tissue Culture Regeneration of *Hibiscus sabdariffa* L. (Roselle)

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Abstract: *Hibiscus sabdariffa* L. (Roselle) has gained popularity as an ornamental, medicinal, industrial and food plant. These industries rely on the fast and guaranteed supply of clones to be cost-effective. Two vegetative propagation methods for *H. sabdariffa* L. are proposed as a technique of ensuring maximum genetic stability. Softwood and semi hardwood cuttings from two-month-old plants were rooted on a medium containing soil, compost and rocksand after dipping in IBA (indole-3-yl-butyric acid) or NAA (a-naphthalene acetic acid) at (0-1.0g/l). Rooting was significantly ($P< 0.05$) affected by the type of cutting and the concentration of auxin used. Softwood cuttings responded more positively to auxin treatment and lower levels (0.5g/l) of auxin stimulated leaf and root formation. Rooting seemed to be more effective in cuttings treated with auxins than untreated cuttings. Regeneration by tissue culture proved to be more successful by using nodal explants. Multiple shoots were initiated on Murashige and Skoog 1962 (MS 1962) medium supplemented with various levels (0-2.0mg/l) of 6-benzyl amino purine (BAP) and kinetin (KIN). Individual shoots with a minimum of two nodes were excised and rooted on MS (1962) medium containing 1.5-2.5mg/l. Regenerants were acclimatized on a mixture (1:1) of sterile peat and soil. They showed vigorous shoot growth (within 3 weeks) and after 5-6 months were suitable for field planting.

Key words: *Hibiscus sabdariffa* L. . roselle . stem cutting . tissue culture . cytokinins . auxins

INTRODUCTION

H. sabdariffa L. (Roselle), an annual shrub, is native of Africa and is cultivated in many tropical and subtropical regions of the world for stem fibers, paper pulp or edible calyces, leaves and seeds. In some countries its flowers are also used for decorative purposes [1]. The medicinal attributes of *H. sabdariffa* L. have also been reported [2-5]. In Mauritius, this plant is mainly recognized as being an invasive weed and only a few people use the flower calyces to make jams and pickles. With the drop in sugar prices in Mauritius, Roselle is now regarded as a new crop with promising potential for intensive cropping systems owing to its multifunctional attributes. Although most ornamental Hibiscus species are vegetatively propagated, Roselle is currently propagated by seeds [2]. Vegetative propagation methods offer many benefits including ability to regenerate clones, convenience and ease of propagation, combination of genotypes and reduction of length of juvenile period [6]. In addition, cuttings remain the most important means of propagating horticultural and ornamental crop species especially ornamental shrubs. If Roselle is to be proposed as an easy growing, alternative crop to farmers, it is crucial to devise a method of rapidly supplying clean and genetically homogenous planting material to them. Traditional methods of vegetative propagation also include micropropagation, whereby new plants are produced under aseptic conditions. Consequently, micropropagation by plant tissue culture offers promising possibilities. Moreover, in recent years, the application of plant tissue culture as a micropropagation technique has become an important biotechnological tool in the multiplication of various plants that have a great economic importance. Likewise, micropropagation techniques offer additional advantages such as the rapid propagation rate, lack of seasonal restrictions, provision of disease free plants, maintenance of self-incompatible inbred lines [7], international exchange of plant materials, culture systems for genetic transformation. Different pathways of regeneration can be adopted in micropropagation. These include direct methods such as axillary bud proliferation and direct organogenesis and indirect techniques involving an intermediate callus phase. Direct methods of plant regeneration usually ensure genetic stability whereas when plant tissues are cultured via callus phase, the plants that are regenerated may exhibit variation [8]. Up to now, research has focused mainly on the propagation of other ornamental Hibiscus species such as *H. syriacus,* [9-10] and *H. rosa-sinensis* [11]. The only report on the propagation of *H. sabdariffa* L. crop relates to the effect of temperature on seed germination [12]. Tissue culture studies on *H. sabdariffa*, L. have involved anthocyanin production in

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callus cultures [13] and genetic transformation of the crop [14]. The use of shoot apices to micropropagate *H. sabdariffa* has been described [15]. Conversely, tissue culture studies on other Hibiscus species have been widely reported [16-22]. Incidentally, all these authors describe organogenesis from the same type of explant namely shoot apices. In this work we suggest two methods of vegetative propagaton of Roselle namely stem cuttings and micropropagation by direct and indirect organogenesis through the use of nodal explants.

MATERIALS AND METHODS

Stem cuttings: Cuttings were taken from two-month-old H. sabdariffa L. (Roselle) plants cv. 'Local' from the farm of the University of Mauritius early in the morning. Healthy branches were randomly selected for the excision of cuttings from the field. Branches were separated into 17-22cm long/ 4mm diameter soft wood and 18-20cm long /6mm diameter semi-hard wood cuttings with 45° slanting cut. All shoot tips were removed and all cuttings were made up of one newly formed leaf with three nodal segments. All cuttings were dipped in 0.05%w/v Dithane M45® fungicide solution prior to auxin treatment. The rooting medium consisted of 50% soil (Low Humic Latosols), 33% compost and 17% rocksand in cylindrical black polyethylene potting bags of 40 x 27 cm dimensions.

Cuttings were dipped for five minutes in 0.5-1.0g/l IBA, (BHD Limited Poole, England) and 0.5-1.0g/l NAA, (Sigma, Aldrich) and were planted to a depth of 2cm in moist rooting medium. Controls were dipped in distilled water. The rooting medium was kept moist throughout the experiment.

***In vitro* regeneration:** Establishment of aseptic seedlings: Mature Roselle seeds were washed in running tap water containing one drop of Tween 20 for 10 minutes and were rinsed three times in sterile distilled water. After an overnight soak in sterile distilled water, seeds were disinfected in a mixture of 0.1% Benomyl® and 0.07% Dithane M45® for 10 minutes followed by rinsing three times in sterile distilled water. The seeds were then dipped into 95% ethanol for 10 seconds and treated with 1% sodium hypochlorite with 2 drops of Tween 20 for 15 minutes followed by thorough rinsing in sterile distilled water for five times. The seeds coats were aseptically removed (manually, under laminar flow, using sterilized scalpels). Seeds were germinated on Murashige and Skoog (MS 1962) [23] medium containing 3% sucrose and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 120°C and 0.138MPa for 20 minutes.

Direct organogenesis: 0-0.5cm nodal segments excised from *in vitro* germinated seedlings were sub-cultured on MS (1962) media containing various concentrations (0.1 mg/l-2.0 mg/l) of 6-Benzyl Amino Purine (BAP) (Sigma, Aldrich) and Kinetin (KIN) (Sigma, Aldrich). The number of leaves that developed from the shoots in the different BAP and kinetin cultures was recorded every week. Shoots were rooted on MS (1962) medium supplemented with 1.5 mg/l-2.5 mg/l of (Sigma, Aldrich). The percentage (%) number of shoots that developed roots was recorded.

Indirect organogenesis: Calli were induced using the leaves and stems from *in vitro* germinated seedlings as explants. Explants of 0.5 × 0.5 cm were inoculated on MS (1962) medium supplemented with Thidiazuron (TDZ) or 2,4-dicholrophenoxy acetic acid (2, 4-D) at 0.1mg/l to 2.0 mg/l and 0.01 mg/l to 0.05 mg/l respectively. One explant was cultured per jar and callus production was recorded as a percentage of explants response. The effect of (4.0-5.0 mg/l), 6-Benzyl amino-purine (BAP) (0.5-2.0 mg/l) and TDZ (0.l-1.5 mg/l) was compared for shoot regeneration from the calli.

Acclimatization of the regenerated plantlets: Regenerated plantlets were acclimatized on a mixture (1:1) of sterile peat and soil in black polythene plastic pots.

Culture conditions and Media: All *in vitro* work was performed on MS (1962) medium containing 3% sucrose and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 120°C for 20 minutes. The cultures were incubated in a culture room at a temperature of 25 ± 2^0C and a photoperiod of 16 hours. The light intensity that was provided to the cultures was 2000 Lux and the relative humidity was 23.5%.

Experimental design and measurements: Ten replicates were used for each treatment in the cutting experiment. Measurements were recorded over period of 4 weeks and each treatment was replicated five times by using a completely randomized design. At two weeks intervals, 3 out of the 5 cuttings per treatment were up rooted carefully, to record the fresh weight, cutting diameter, root number, number of branches, stem and root dry weight. Collected data was treated by ANOVA, variations and interaction effects were compared using the Dunnett's test at the 5% level of significance using MINITAB 13.1 and Microsoft Excel 2007 software.

For the *in vitro* experiment, all treatments were replicated five times using a completely randomized design. The number of leaves that initiated from each shoot was noted. The percentage number of shoots that

developed roots during the experiment was also recorded. Collected data were analyzed by one-way ANOVA and deviations among means were evaluated using the Minitab 13.1 statistical software at P=0.05. Least significant differences (LSD) were computed at the 5% level of significance to compare the treatment means.

RESULTS

Stem cuttings

Number of leaves: The number of leaves increased significantly with time in both softwood and semi-hardwood cuttings on the different treatments. However, a

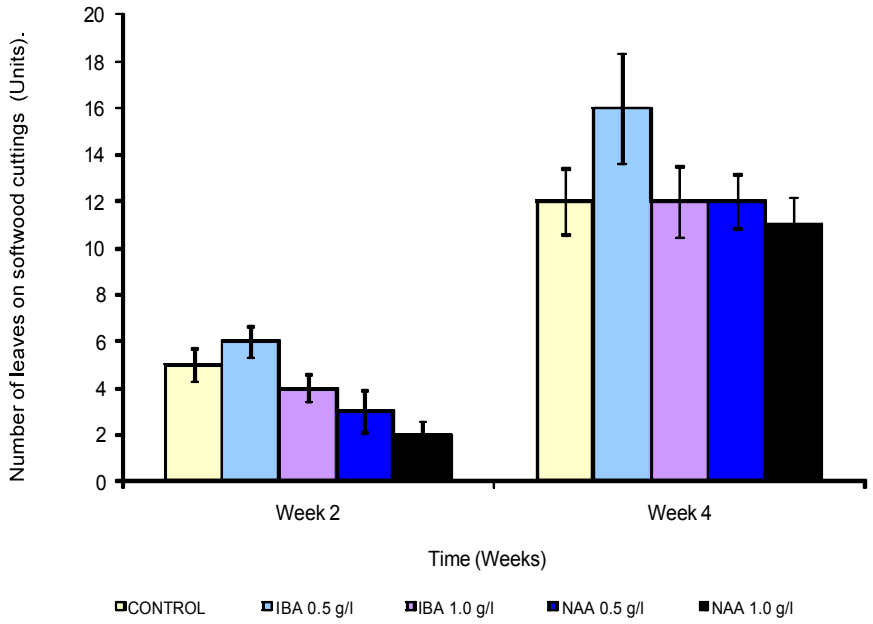

Fig. 1: Number of leaves on softwood cuttings. Vertical bars indicate±standard error (±SE) of means (n=3)

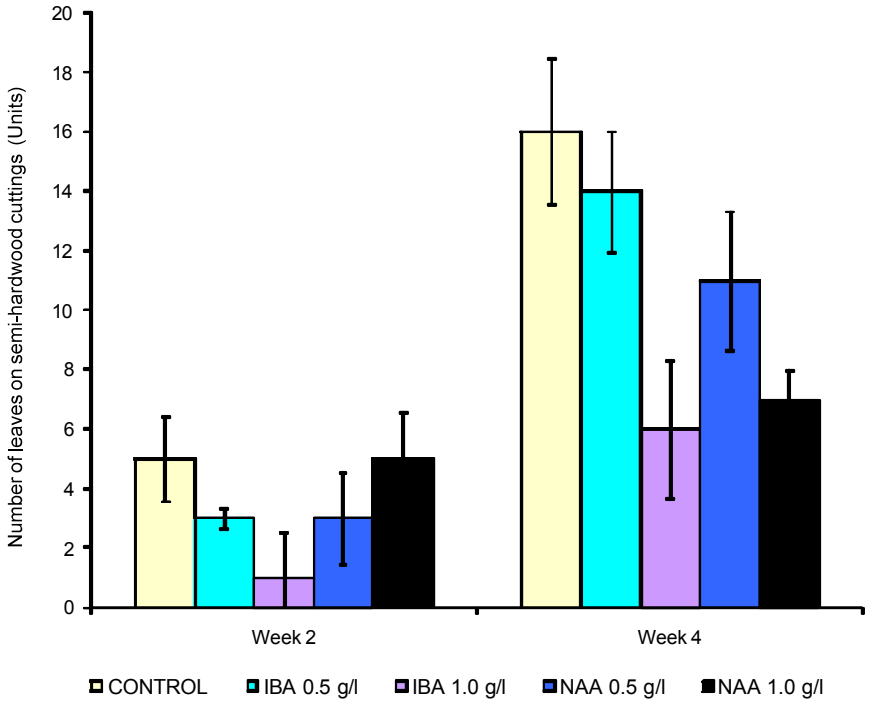

Fig. 2: Number of leaves on semi hardwood cuttings. Vertical bars indicate±SE of means (n=3)

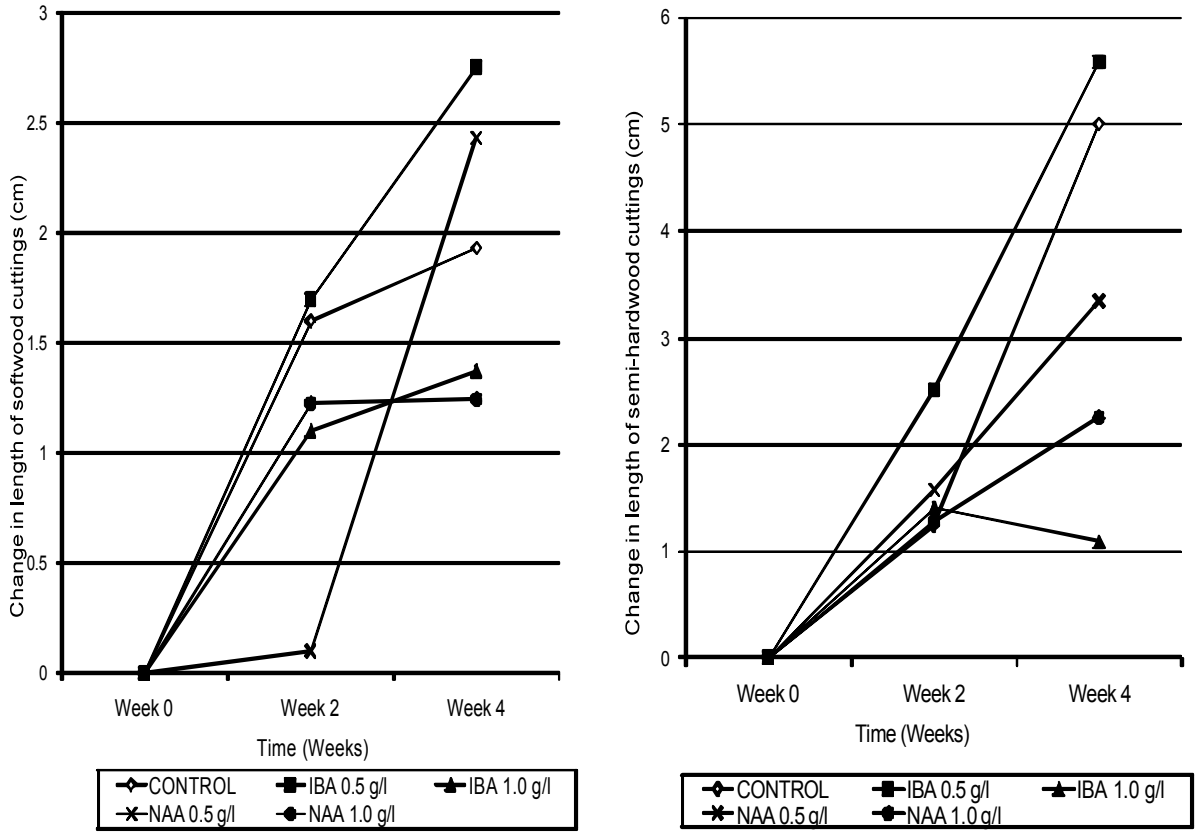

Fig. 3: Change in length of softwood and semi-hardwood cuttings. Initial length of softwood and semi-hardwood cuttings were 19.57±2.02cm and 19.42±1.96cm respectively. LSD at 5% level of significance to compare any two treatment means = 3.02

greater ($P<0.05$) increase in leaf number was noted in the case of 0.5 g/l IBA for softwood cuttings whereas the control treatment gave the highest number of leaves after 4 weeks in the case of semi-hardwood cuttings (Fig. 1 and 2).

Length of cuttings: Data analysis showed that change in length of cuttings in both softwood and semi hardwood cuttings was significantly ($P<0.05$) influenced by the level and type of plant growth regulators (PGRs) used. As such, softwood cuttings showed a general increase in length for all treatments, with significantly larger increases on 0.5g/l IBA. However, in the case of semi-hardwood cuttings, there was a much greater increase in length in all treatments, with significantly larger increases on 0.5g/l IBA (Fig. 3). For both types of cuttings, the overall change in length was smallest when higher levels (1.0g/l) of PGRs were used.

Diameter of cuttings: All the treated cuttings showed a significant ($P<0.05$) increase in diameter with time irrespective of the treatments used (Fig. 4). Change in diameter of cuttings was also significantly ($P<0.05$) affected by the treatment employed. Conversely the increase in diameter was significantly much lower in the case of the control treatments for both softwood and semi hardwood cuttings. 0.5g/l IBA and/or NAA gave significantly ($P<0.05$) higher increase in diameter than the higher levels of auxins (1.0g/l), as depicted in Fig. 4.

Root number: Both softwood and semi-hardwood cuttings produced roots in all treatments (Fig. 5a and 5b). The number of roots was significantly affected by the concentration of auxin used ($P<0.05$). The interaction effect between the type of cutting and treatment on root number was also significant ($P<0.05$). For softwood cutting, the highest increase in root number was obtained with 1.0g/l IBA and the lower concentration of IBA (0.5g/l) gave a superior number of roots in semi hardwood cuttings. The highest number of roots at the end of the experiment was 25 for softwood cuttings and 20 for semi-hardwood cuttings.

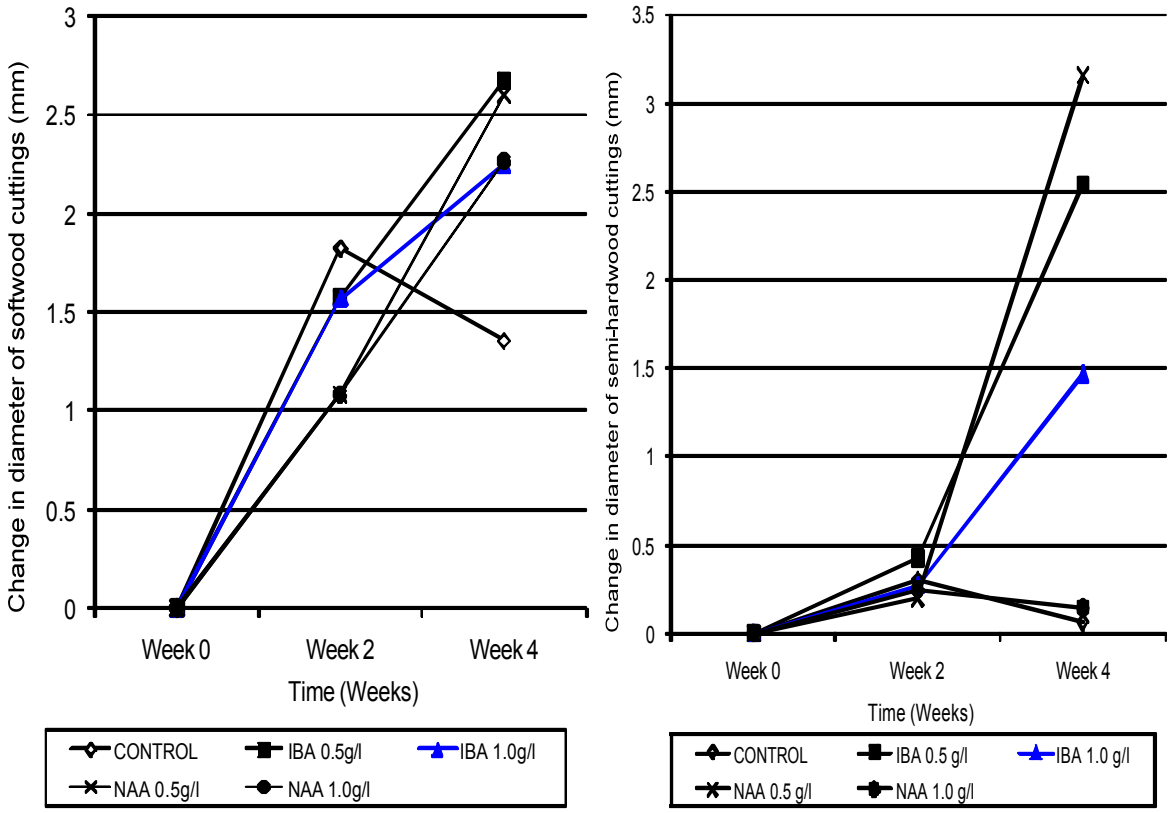

Fig. 4: Change in diameter of softwood and semi-hardwood cuttings. Initial diameter of softwood and semi-hardwood cuttings were 3.90±0.05 mm and 6.67±0.10 mm respectively. LSD at 5% level of significance to compare any two treatment means = 2.35

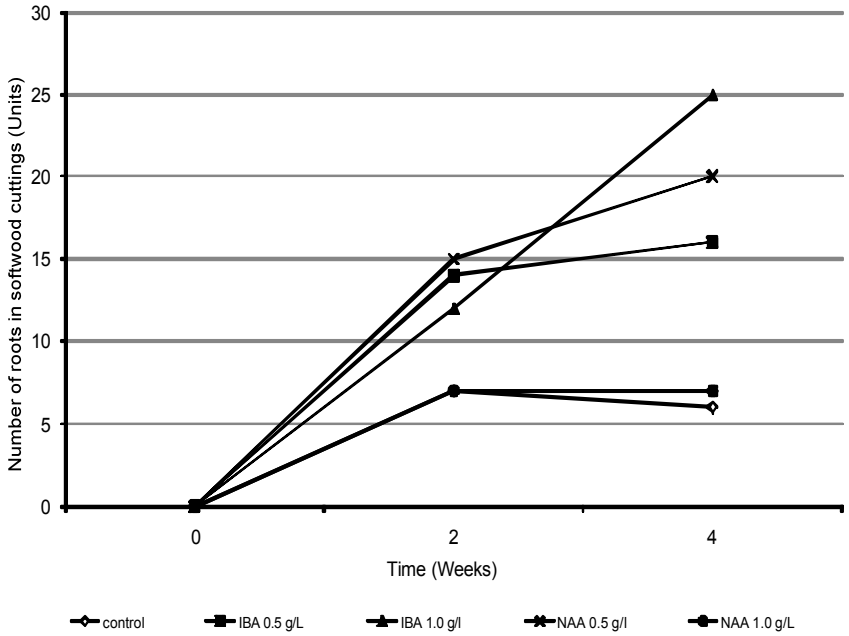

Fig. 5a: Effect of IBA and NAA on the root number of softwood cuttings. LSD to compare any two treatment means = 7.81

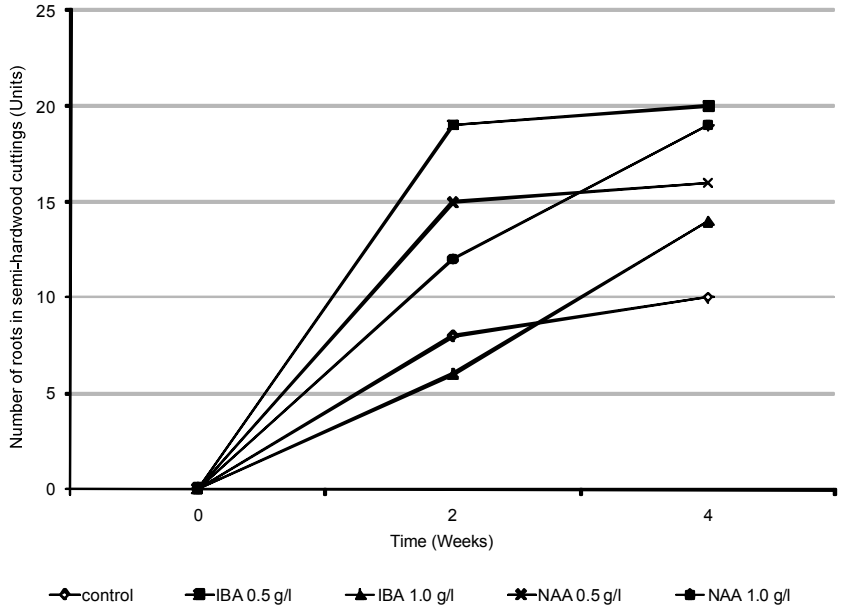

Fig. 5b: Effect of IBA and NAA on the root number of semi-hardwood cuttings. LSD to compare any two treatment means = 7.80

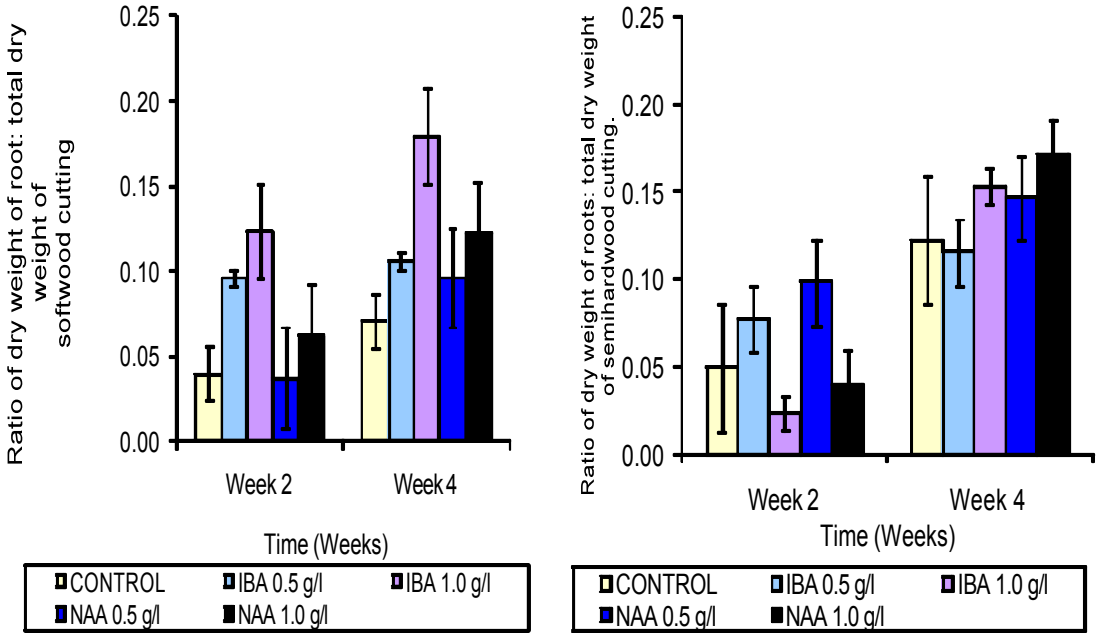

Fig. 6: Effect of IBA and NAA on the ratio of dry weight of roots: total dry weight of cutting

Dry matter accumulation: Auxin treatment was seen to have a significant effect ($P<0.05$) on dry matter accumulation depending on the type of cutting used. As such, dry weight of cutting increased significantly in both softwood and semi hardwood cuttings treated with 0.5g/l and 1.0g/l IBA compared to other treatments during the first four weeks after planting (Fig. 6.).

***In vitro* regeneration**

Direct organogenesis: Table 1 illustrates the number of elongated shoots (expressed as a percentage) that were initiated from axillary buds of *in vitro* germinated seedlings of H. sabdariffa on MS (1962) medium supplemented with various levels of BAP. It can be noted that 81% of shoots were produced in the hormone free

Table 1: Shoot regeneration from axillary buds of *H. sabdariffa* on MS+BAP

Conc. of BAP (mg/l)	Shoot formation (%)	No. of shoots per nodal explant
0	81.0±5.32[b]	3.2±0.22[b]
0.1	73.0±2.54[b]	1.4±0.19[b]
0.5	46.2±2.07[a]	0.8±0.19[a]
1.0	39.6±1.59[a]	0.6±0.14[a]
1.5	39.6±1.13[a]	1.0±0.02[a]
2.0	33.0±1.12[a]	1.2±0.07[a]

Means±S.E followed by the same letter in a column are not significantly different, as indicated by the LSD test (P=0.05)

Table 2: Shoot regeneration from axillary buds of *H. sabdariffa* on MS+KIN

Conc. of KIN (mg/l)	Shoot formation (%)	No. of shoots per nodal explant
0	100±5.32[b]	3.0±0[b]
0.1	82.0±1.90[b]	1.8±0.12[b]
0.5	35.2±1.20[a]	1.3±0[a]
1.0	25.0±0.88[a]	1.6±0.14[a]
1.5	19.6±0.94[a]	1±0.02[a]
2.0	18.0±0.12[a]	1.0±0[a]

Means±S.E followed by the same letter in a column are not significantly different, as indicated by the LSD test (P=0.05)

medium while only 33% of shoots resulted in the 2.0mg/l of BAP respectively.

The hormone also had an effect on the initiation of elongated (normal) and stunted shoots. Interveinal chlorosis, which is yellowing of the leaves between the veins, was observed in cultures that developed normal shoots (Plate 1a) as well as in cultures that developed stunted shoots (Plate 1b). The development of the normal shoots started with the formation of a small mass of callus at the cut ends but after elongation no callus was found at the base.

Table 2 indicates the% and the number of shoots that were initiated on medium with Kinetin. MS medium supplemented with 0.1 mg/l of kinetin and MS (1962) medium without any hormone gave a higher response in the shoot initiation.

The effect of KIN was such that it contributed largely to the regeneration of the shoots (normal and stunted) rather than to the formation of roots. Moreover, the roots that were initiated from the cultures were not profusely developed (Plate 1b).

Microshoots that attained a height of 2-3 cm with at least one node were individually excised from the shoot cluster and selected for rooting. Rooting was seen to be significantly more efficient with increasing concentration

Table 3: % Root initiation on MS+ IBA

IBA (mg/l)	Root formation (%)
1.5	28.1±0.6[c]
2.0	59.6±1.4[b]
2.5	86.2±0.0[a]

Means±S.E followed by the same letter in a column are not significantly different, as indicated by the LSD test (P=0.05)

Plate 1a: Shoot formation from *H. sabdariffa* explants on MS+KIN after 8 weeks

Plate 1b: Shoot & Root formation from *H. sabdariffa* explants on MS+KIN after eight weeks

of IBA; hence the highest rooting percentage was obtained at 2.5mg/l IBA (Table 3). The regenerated plantlets were successfully acclimatized in oven-sterilized soil under 100% humidity after two weeks (Plate 2.).

Callus initiation: Callus formation was observed on both TDZ and 2,4-D on leaf explants of *H. sabdariffa* (Plate 3).

Table 4: % Callus formation on leaf explants of *H. sabdariffa* using TDZ and 2,4-D

Conc.of TDZ mg/l	Callus formation (%)	Conc of 2,4-D(mg/l)	Callus formation (%)
0.1	20	0.01	80
0.5	60	0.02	80
1.0	60	0.03	80
1.5	80	0.04	80
2.0	60	0.05	80

Plate 2: *H. sabdariffa* plantlet on a mixture (1:1) peat and soil

Plate 3: Callus initiation from leaf explants of *H. sabdariffa* cultured on TDZ

However, 1.5 mg/l TDZ showed a higher response in callus formation (80%) (Table 4); nevertheless the lowest callus formation was observed with 0.1 mg/l of TDZ. On the other hand, all levels of 2,4-D (0.01-0.05mg/l) produced equally high percentage of callus (80%). Conversely, these calli could not be regenerated further as only profuse chlorophyll formation (organogenic calli) was observed after two weeks culture on different levels of KIN (0.1-5.0mg/l).

DISCUSSION

Stem cuttings: This study demonstrates that *H. sabdariffa* can be propagated through the use of both softwood and semi hardwood cuttings especially by using auxin treatment. However, based on the number of leaves produced after four weeks, it seems that softwood cuttings are more responsive to auxin treatment than semi hardwood cuttings of *H. sabdariffa*. Several factors can affect the number of leaves produced including the type of cuttings used; the plant growth regulators utilised, temperature, dry matter content of the cuttings before sticking in the medium and the health status of the plant [24]. It seems however that compared to other hibiscus species, *H. sabdariffa* L. produced new leaves faster (than some other Hibiscus species such as *H. rosa sinensis* (27 days) [25]. In our experiment, after 14 days, Roselle already had 3 fully expanded leaves in semi-hardwood cuttings and 4 in soft wood cuttings.

The leaves produced in the experiment varied with the treatment given and the type of the cuttings used. Fig 1 and Fig 2 also indicate that the amount of dry matter originally present in the semi hardwood cuttings had a more significant effect than that of the auxin treatment as the semi hardwood cuttings had an initial weight of 1.512±0.366g and the softwood cuttings were lighter (0.677±0.133g). The number of leaves produced is also determined by the initial amount of dry matter in the cuttings [11, 26]. The number of leaves in the cuttings is very important for successful post propagation. As such, softwood cuttings produced a total of 16 leaves cumulatively for the 4 weeks period with 0.5g/l IBA whereas the semi-hardwood cuttings produced only 14 leaves. For the case of semi hardwood cuttings, the highest number of leaves was produced in the control. Softwood cuttings seemed to respond more positively to auxin treatment than semi hardwood with respect to the number of leaves.

Similarly, both softwood and semi-hardwood cuttings witnessed the highest increase in length and diameter with lower levels of auxin (0.5g/l IBA & 0.5g/l NAA). The increase in length of cuttings and increase in diameter seemed to be due to the use dry matter for the shoot growth. The smallest increase in diameter was observed with 1.0g/l NAA in both types of cuttings probably due to the diversion of the cuttings food reserves towards root formation [27]. Auxin treatment had a significantly positive effect on root number of *H. sabdariffa* cuttings as the untreated cuttings produced a lower number of roots in both species after four weeks.

The treatment giving the highest dry matter accumulation in the roots was IBA 1.0g/l for softwood and semi hardwood cuttings.

Root production in *H. sabdariffa* was faster (within two weeks) than in *H. rosa-sinensis* L. cultivar 'Paramaribo' which took 21 days to produce the first visible roots [11]. The effect of various co-factors including endogenous auxins on rooting has been formerly reported [6, 28-29] and also seem to be species dependent [28]. As such, although the same auxin (IBA) produced similar number of roots in both types of cuttings, it seemed that, softwood cuttings required a higher concentration of IBA for maximum root production (1.0g/l). This could be due to dry matter content which was higher in semi hardwood cuttings batch initially and at the end of the experiment. The effect of initial carbohydrate content (dry matter) on rooting ability has previously been described [30-31].

***In vitro* regeneration:** *In vitro* regeneration from H *sabdariffa* seemed to be more successful by axillary bud regeneration than by indirect organogenesis. As such more efficient shoot regeneration from axillary buds of *H. sabdariffa* was obtained on MS media with low levels of cytokinins than on media supplemented with higher concentrations of BAP or KIN. The optimum concentration of cytokinin for successful shoot regeneration was between 0-0.1mg/l for both growth regulators. These findings are in accordance with those of Samanthi *et al*. [20], on tissue culture of *H. cannabinus* whereby the optimum concentration of BAP was 1.98mg/l for shoot regeneration from nodal explants with negative results on using higher cytokinin concentrations. Similar type of response was published by Jorge *et al*. [32] who also reported that cytokinin is directly responsible for reprogramming apical meristem axes of cotton towards the multiplication of buds and also fewer shoots with higher dose of growth regulators were observed. In the present study, we also found that kinetin was more responsive than BAP in inducing shoot formation in contrast to the findings of Christensen *et al*. [33] who obtained efficient shoot regeneration from nodal cuttings of *H. rosa sinensis* L. on 0.5mg/l BAP. The incidence of abnormal shoots and interveinal chlorosis during tissue culture of hibiscus species has formerly been reported by Samanthi *et al*. [20] on *H. cannabinus* and on *H. rosa sinensis* by Christensen *et al*. [33]. Our study shows that single node explants can also be used for the *in vitro* regeneration of Roselle even with very low levels or even in the absence of cytokinins. Moreover, our results differ from the report of Gomez-Leyva *et al*. [15], who used shoot apices with cytokinins to induce sprouting in that we have made use of different explants. We infer that single nodes probably do not suffer from the apical dominance effect exerted by the apical meristem and hence were able to sprout without or with very low levels of growth regulators. Likewise, Gomez-Leyva *et al*. [15] highlighted the significant role of BAP and m-topolin in reprogramming the cells in shoot apices of Roselle towards sprouting.

In vitro rooting of initiated shoots was efficiently obtained after a period of 8 weeks either on MS medium containing 2.5mg/l IBA. However, simultaneous rooting was observed also on shoots cultured in both shoot initiation and shoot growth media (MS+Cytokinin). The positive effect of IBA on rooting of *in vitro* regenerated shoots has been widely reported [34-36].

Indirect organogenesis from leaf explants of *H. sabdariffa* proved to be more intricate in that although callus formation was observed on both TDZ and 2,4-D supplemented medium, these calli could not be regenerated. Our results confirm the significant effect of genotype in regeneration by tissue culture methods [37-40]. Similarly other Hibiscus species namely *H. syriacus* could be efficiently regenerated from nodal explant on MS media supplemented with TDZ [41]. Likewise, Jenderek *et al*. [19] have succeeded in regenerating *in vitro* derived callus from *H. syriacus* seedling fragments on MS medium supplemented with BAP or KIN.

CONCLUSIONS

This study has shown for the first time that *H. sabdariffa* can be propagated by vegetative methods both *in vitro* and ex vitro. Although auxins were not a *sine qua non* condition for effective rooting of softwood or semi hardwood cuttings, the use of exogenous auxins accelerated and ensured uniformity of the rooting process. The present findings indicate also the possibility of using micropropagation methods for the *in vitro* regeneration of *H. sabdariffa*. The use of direct organogenesis as suggested in this work proved to be a quick and efficient method for *in vitro* regeneration of *H. sabdariffa*. In addition, by making use of single node explants, the number of micropropagules used in one tissue culture multiplication cycle offer the advantage of being more abundant than through the use of shoot apices. The fact that no or low levels of cytokinins induced higher sprouting efficiency also reduces the risks of somaclonal variation as a result of the use of plant growth regulators in tissue culture.

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