

RESEARCH ON *IN VITRO* SHOOT PROLIFERATION OF *Stemnona tuberosa* L.

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ABSTRACT

Stemnona tuberosa Lour. (Stemonaceae) is known as an antitussive medicinal plant in traditional medicine. Recently, its tuberous roots have been seriously exploited for medicinal materials in Vietnam. Therefore, it is necessary to conduct research on the propagation of *S. tuberosa* by tissue culture for its conservation and rapid production. In this study, the strong positive influence of coconut water (CW) on *in vitro* shoot proliferation and the effective shoot formation in *S. tuberosa* was recorded. MS medium supplemented with 1.5 mg/L BAP, 30 g/L sucrose, and 10% CW was determined as the optimal medium for the shoot multiplication of *S. tuberosa* shoots from the nodal explants. On that medium, 8.87 effective shoots per explant were obtained.

Keywords: *Stemnona tuberosa*, proliferation, BAP, explant, coconut water.

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1. INTRODUCTION

“Bach bo” is a crucial medicinal plant with the scientific name *Stemnona tuberosa* Lour., which belongs to the family Stemonaceae. Its tuberous roots are rich in alkaloids such as stemonine, tuberostemonine and neotuberostemonine [1] and therefore has been recorded as an antitussive medicinal plant in Vietnamese pharmacopeia [2]. Currently, there are many herbal products containing ingredients from the tuberous roots of *S. tuberosa*. The natural material source of *S. tuberosa*, therefore, has been significantly declining, not only in Vietnam but also in its distribution places around the world. Demand recently for high-quality seedlings of medicinal plants in general, and of *S. tuberosa*, in particular, ensure the standards and traceability which are required

by the Ministry of Health in the making process of herbal products is on the rise in Vietnam. Research on micropropagation of *S. tuberosa* - a valuable medicinal plant is essential for conservation and cultivation.

Protocols for *in vitro* culture of *S. tuberosa* have been established in Bangladesh and India, in which the MS medium was used as the basic medium for the culture. In those protocols, plant growth regulators used for multiplication purposes were high in concentration and it is noticed that there were no correlations or agreements between the publications [3, 4, 5]. Although the type of explants of *S. tuberosa* were the same, the differences in age and characteristics of donor plants, as well as different collection seasons could be the causes of these differences [3, 4, 5]. To produce large-scale plantlets with all the inherited traits of the donor plant, we carried out *in vitro* regeneration of *S. tuberosa* from nodes of the selected domestic 4-year-old

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mother plant. In this paper, we present the results on the investigation of plant growth regulators, coconut water, and sugar content on *in vitro* shoot proliferation to establish an efficient micropropagation protocol for *S. tuberosa*. The results can be applied for a large-scale production of *S. tuberosa* plantlets for cultivation in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

The juvenile nodes (1,0 -1,5 cm) of the 4-year-old *Stemona tuberosa* were collected during Spring season in Thanh Hoa province were used as the explants.

2.2. Methods

Explants were cleaned under a running water tap for 20 min to remove dust, and then they were shaken gently in 1% detergent powder for 10 min and washed for 4-5 times with distilled water. In a laminar, explants were treated with 70% ethanol in a sterile flask for 30 sec, following 8 – 10 min surface sterilized with 0.1% HgCl₂ to determine the appropriate period for sterilization of *S. tuberosa* nodal explants. After sterilization, the two ends of the explants were dissected and discarded (the wounded tissue which directly contacted with HgCl₂ solution) and explants

were inoculated on MS medium supplemented with 0.5 mg/L BAP and 30 g/L in test-tubes (22 x 200 mm).

The nodal segments of the regenerated shoots were fortified with MS medium supplemented with BAP (0 - 2.0 mg/L), NAA (0 - 0.3 mg/L), IBA (0 - 0.3 mg/L) and sucrose (0 - 60 g/L), coconut water (0 - 20%) at different concentrations and combinations to explore the optimal medium for proliferation.

pH of the medium was adjusted to 5.7 - 5.8 before dispensing in a flask and autoclaving at 121°C and 1.1 kg/cm² for 20 min. Inoculation room was maintained at 25 ± 2°C, < 70% in humidity, illuminance level of 2,000 - 3,000 lux, 16 hours of light a day. The experiments were completely randomized design. Each experiment was repeated thrice using 15 replicates. The data were subjected to Analysis of Variance (ANOVA) for one factor at P ≤ 0.05 per experiment. The significant difference between the means was compared with Duncan's Multiple Range Test by the program SPSS 20.

3. RESULTS AND DISCUSSION

3.1. Effect of duration treatment of 0.1% HgCl₂ on surface sterilization of *S. tuberosa* explants

Table 1. Effect of duration treatment of 0.1% HgCl₂ on *S. tuberosa* explants

Duration* (min)	Survival percentage** (%)	Contaminated percentage** (%)	Death percentage** (%)
8	46.67	46.67	6.67
10	83.33	10.00	6.67
12	53.33	6.67	40.00

*Culture medium: MS + 30 g/L sucrose + 0.5 mg/L BAP

** Data indicate the mean of three replicates of the experiment with ten explants used per replication.

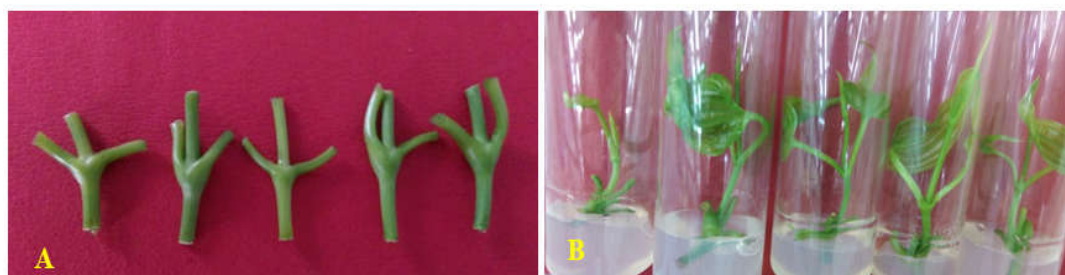


Figure 1. Surface sterilization of *S. tuberosa* explants. A: the juvenile nodal segments of *S. tuberosa* explants; B: The explants of *S. tuberosa* 4 weeks after sterilization with 0.1% HgCl_2 for 10 min.

Surface sterilization is the main aspect of tissue culture to prevent it from contamination, to obtain sterile plant material is difficult because during sterilization the microorganism should be eliminated, while the living materials should be survived. In this experiment, we investigated the effect of the duration of HgCl_2 0.1% treatment on *S. tuberosa* explants. After 4 weeks of culture, the percentage of explant survival and microbial contamination was observed.

The results in table 1 showed that, the longer the exposure time of the explants to HgCl_2 solution, the lower the rate of bacterial contamination (6.67%, 12 min). However, when explants exposed longer time to HgCl_2 , the explants turned brown and eventually died. The percentage of browning and dying explants was quite high (up to 40%) as the exposure time of 0.1% HgCl_2 was 12 min.

In this experiment, we have succeeded in controlling the contamination, determining that 10 min is the optimal exposure time of 0.1% HgCl_2 to the juvenile nodal segments of *S. tuberosa*.

3.2. Effect of BAP on *in vitro* shoot proliferation of *S. tuberosa*

In previous studies on *in vitro* shoot multiplication in *S. tuberosa* indicated that BAP, Kin, and TDZ were suitable for

proliferation. However, those results also showed that the different combinations and concentrations of these substrates brought the various number of regenerated shoots [3, 4, 5]. BAP is considered to be one of the effective cytokinins for induction of organogenesis and thereby it enhanced the ability of micropropagation in many plant species. In the present study, we investigated the effect of BAP concentration (0-2 mg/L) on the number of shoots and on the growth of shoots of *S. tuberosa* explants. The given results showed that the percentage of multiple shoot formation reached 73.33% in treatment 0.5 mg/L BAP, and the maximum of this rate (100%) was obtained from the third treatment (1.0 mg/L BAP) onwards (Table 2). It is clearly indicated that the increase of BAP concentration has increased the percentage of multiple shoot formation, as well as the number of new shoots per the nodal explant. The average number of new shoots was significantly influenced by different concentration of BAP. It increased from 2.43 to 4.63 shoots/explant according to the increase of BAP concentration (0.5 mg/L - 2.0 mg/L) in the culture medium. The maximum number of shoots obtained at the highest concentration of BAP in the experiment. Their regenerated shoots, however, were less vigorous than the others (Table 2, Figure 2).

Table 2. Effects of various concentrations of BAP on *in vitro* shoot proliferation in *S. tuberosa*

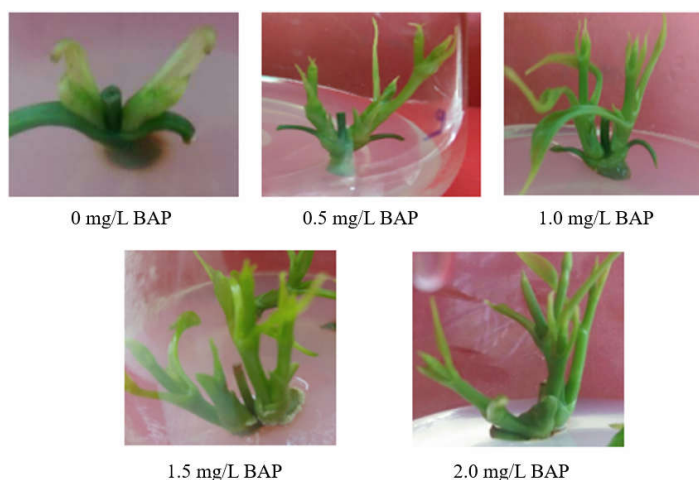
BAP* (mg/L)	Percentage of multiple shoot formation	No. of shoots**	Length of the shoots** (cm)	Vigor of shoots
0,0	-	-	-	
0.5	73.33	$2.43^c \pm 1.09$	$2.39^c \pm 0.92$	++
1.0	100	$2.82^b \pm 0.81$	$3.06^{bc} \pm 0.69$	++
1.5	100	$4.57^a \pm 0.56$	$3.82^a \pm 0.43$	++
2.0	100	$4.63^a \pm 1.16$	$3.28^b \pm 1.02$	+
		P < 0.05	P < 0.05	

* Supplemented to MS + 30 g/L sucrose (Control medium)

** Data indicate mean $\pm$ SD; mean followed by the same letter was not significantly different by Duncan's test at 0.05% probability.

Fifteen replicates were used per treatment and the experiment was repeated thrice.

+++ green leaves, short internodes; ++ pale green leaves, long internodes; + small leaves, long internodes;

Figure 2. Effect of BAP concentration on *in vitro* shoot proliferation in *S. tuberosa*

In terms of the analysis results, the number of shoots in 1.5 mg/L and 2.0 mg/L BAP treatments were the same rank according to Duncan's test (Table 2). The length and vigour of shoots in 1.5 mg/L BAP treatment, however, were better than another one. Besides, the change of their shoot length was a significant difference. In previous studies of *in vitro* propagation in *S. tuberosa*, the percentage of shoot multiple responses was quite low. At the same concentration range of BAP, the number of

shoots per explant was also lower than this result, only got under 50% in percentage response with 1.00 shoot/explant. This might be due to the differences of genetic resources or the origin of the used chemicals. No multiple shoot formation occurred in plant growth regulators free medium after 6 weeks of culture.

Therefore, we concluded that 1.5 mg/L of BAP was the most suitable concentration for the following experiments of *in vitro* shoot proliferation.

3.3. Effect of BAP in combination with auxin (IBA, NAA) on *in vitro* shoot proliferation

Normally cytokinin inhibits the root induction and enhances the shoot proliferation and elongation during *in vitro* propagation of plants. However, many studies have demonstrated that lower concentrations of auxins in combination with higher concentrations of cytokinin enhance the rate

of shoot proliferation in micropropagation of plants [4, 6]. The purpose of this experiment was to increase the number of shoots per explant, hence, we developed the hormone combined treatments to determine the effects of 1.5 mg/L BAP in combination with two concentrations at 0.1 and 0.3 mg/L of IBA and of NAA. Follow-up results after 6 weeks are presented in table 3.

Table 3. Effect of combination 1.5 mg/L BAP and IBA, NAA (at 0.1 mg/L, 0.3 mg/L) on *in vitro* shoot proliferation in *S. tuberosa*

Growth regulators (mg/L)			Percentage of multiple shoot formation	No. of shoots	Length of shoots (cm)	Vigor of shoots
BAP	NAA	IBA				
1.5	-	-	100	4.58 ^a ± 0.56	3.82 ^a ± 0.43	++
1.5	0.1		100	4.26 ^a ± 0.25	3.33 ^{ab} ± 0.17	++
1.5	0.3		86.67	3.05 ^b ± 0.72	2.82 ^{bc} ± 0.53	+
1.5		0.1	86.67	2.78 ^c ± 0.46	3.00 ^b ± 0.31	+
1.5		0.3	56.67	2.62 ^d ± 0.91	2.07 ^c ± 0.14	+
				P < 0.05	P < 0.05	

*Supplemented to MS + 30 g/L sucrose (Control medium)

**Data indicate mean±SD; mean followed by the same letter was not significantly different by Duncan's test at 0.05% probability.

Fifteen replicates were used per treatment and the experiment was repeated thrice.

+++ : green leaves, short internodes; ++ : pale green leaves, long internodes; + : small leaves, long internodes.

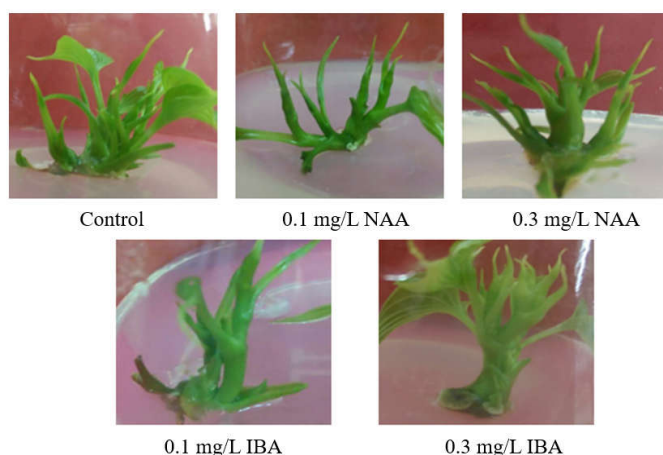


Figure 3. Effect of 1.5 mg/L BAP in combination with auxin (IBA, NAA) on *in vitro* shoot proliferation in *S. tuberosa*

From the data furnished in table 3, we observed that the combination 1.5 mg/L BAP and NAA was more effective than the combination 1.5 mg/L BAP and IBA. However, the value direction of all assessed indicators was gradually decreased with increasing concentrations of NAA and IBA, and the value of the combination 1.5 mg/L BAP and 0.3 mg/L IBA was the lowest in this experiment. The studied results of Biswas *et al.* (2011)[3] on the effect of plant growth regulators on *in vitro* shoot multiplication in *S. tuberosa*, showed that the percentage responses of nodal explants on *in vitro* shoot multiplication were just under 50% on MS medium supplemented with a range of the individual BAP concentration (1.0 - 5.0 mg/L). And they obtained the highest response percentage and the biggest number of shoots per explant, 97% and 12.7 respectively, on

media fortified with 3.0 mg/L BAP + 0.5 mg/L NAA after 60 days of culture [4]. However, our results of the current study seemed opposite to these ones. MS supplemented with 1.5 mg/L BAP alone induced the best response, 100% of the cultured explants gave multiple shoots with average 4.58 shoots, the vigor of regenerated shoots was also the best on it (Figure 3). The opposition might be caused by the different ages of used explants, and our explants spent over two subcultures. In addition, it might be caused by the difference in the composition of previous culture media which were accumulated in the culture cell/tissue causing the different organogenesis.

3.4. Effects of various concentrations (g/L) of sucrose on *in vitro* shoot proliferation in *S. tuberosa*

Table 4. Effects of various concentrations of sucrose on *in vitro* shoot proliferation in *S. tuberosa*

Sucrose (g/L)	Percentage of multiple shoot formation	No. of shoots per explant	Length of shoots (cm)	Vigor of shoots
Control	-	1.07 ^c ± 0.31	2.01 ^b ± 0.73	+
10	76.67	2.33 ^b ± 0.92	3.50 ^{ab} ± 0.07	++
30	100	4.73 ^a ± 0.09	3.48 ^{ab} ± 0.16	+++
50	100	4.57 ^a ± 0.46	3.73 ^a ± 0.23	+++
		P < 0.05	P > 0.05	

* Supplemented to MS + 1.5 mg/L BAP (Control medium)

** Data indicate mean ± SD; mean followed by the same letter was not significantly different by Duncan's test at 0.05% probability.

Fifteen replicates were used per treatment and the experiment was repeated thrice.

+++ : green leaves, short internodes; ++ : pale green leaves/leafless, long internodes; + : small leaves, long internodes.

The addition of an external carbon source to the medium enhances the proliferation of cells and regeneration of green shoots [7]. The optimal sucrose concentration in a medium should be sufficient to satisfy the basic energy requirements for cell division/differentiation and not impose any

negative osmotic effects on shoot formation [8]. This indicates that sucrose acts not only as a carbon energy source in a medium but also as an osmotic and that different concentrations of sucrose are one of the factors controlling the induction and growth of shoots. We found that the vigor of

regenerated shoots in all the above treatments was not well (leafless), although their number of shoots per explant was quite high. Therefore, we accomplished this further experiment with 4 treatments of sucrose concentration to study on the effect of various concentrations of sucrose on *in vitro* shoot multiplication in *S. tuberosa*. The result of this experiment after 6 culture weeks was presented in table 4 and figure 4.

No shoot multiple was formed in a medium without sucrose (Table 4). Although at least one or two axillary shoots might be

regenerated in the non-sucrose medium, their length and vigor were not well. When adding sucrose to the culture media, the percentage of shoot formation, the number of shoots per explant, and the length of shoots, also shoots' vigor increased with the increase of the sucrose concentration in the medium up to 50 mg/L. However, there was statistically no significant difference in all parameters in 30 mg/L or 50 mg/L of sucrose concentration. This implied that the shoot development and proliferation of *S. tuberosa* shoots did not be affected by the osmotic level of the medium.

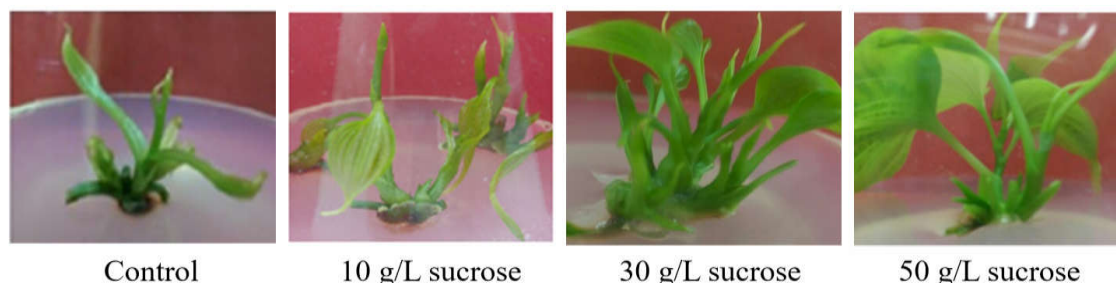


Figure 4. Effect of various concentrations of sucrose on *in vitro* shoot proliferation in *S. tuberosa*

On the other hand, we observed that the appearance of leaves and also the size of leaves were promoted when the sucrose concentration in the medium was greater than equal to 40 g/L (Figure 4). In addition to the propagation rate, the *in vitro* development of shoots should be considered when choosing

the best medium. These observations helped us in choosing of the appropriate sucrose concentration (30 g/L) for the shoot multiplication in *S. tuberosa*.

3.5. Effects of various concentrations of coconut water on *in vitro* shoot proliferation in *S. tuberosa*

Table 5. Effects of various concentrations of coconut water in association with 1.5 mg/L BAP on *in vitro* shoot proliferation in *S. tuberosa*

Coconut water (%)	Percentage of multiple shoot formation	No. of shoots per explant	Length of shoots (cm)	Vigor of shoots
0,0	100	4.37 ^c ± 0.81	3.49 ^c ± 0.92	+++
5	100	5.75 ^b ± 1.13	3.76 ^b ± 0.72	+++
10	100	8.87 ^a ± 0.56	3.86 ^a ± 0.69	+++
15	100	8.43 ^a ± 1.16	3.82 ^{ab} ± 0.43	+++
		P < 0.05	P < 0.05	

*Supplemented to MS + 30 g/L sucrose + 1.5 mg/L BAP (Control medium)

****Data indicate mean $\pm$ SD; mean followed by the same letter was not significantly different by the Duncan's test at 0.05% probability.**

Fifteen replicates were used per treatment and the experiment was repeated thrice.

+++: green leaves, short internodes; **++**: pale green leaves/leafless, long internodes; **+**: small leaves, long internodes

Coconut water (CW) is said to be complex in nature because of its indefinite composition. Efficiency of adding the appropriate concentration of coconut water to media for shoot proliferation and proved in many medicinal plants [9, 10]. Therefore, we carried out the experiment with 4 following treatments such as 0%, 5%, 10%, 15% of coconut water. The goal of this experiment was to evaluate the effects of CW concentration in association with the BAP in the inoculating media to see if it had beneficial effects on the *in vitro* propagation rate and growth of the regenerated shoots in *S. tuberosa*.

Shoot multiplication of *S. tuberosa* in the presence of coconut water was significantly

higher compared to the control containing only 1.5 mg/L BAP after 6 weeks of culture. When CW was augmented in the medium not only the number of shoots increased but also the shoot height was significantly different. Significant differences also existed among the various CW treatments for all parameters studied in the same time period of inoculation. Among the various levels of CW tested, 10% was found optimum as it gave rise to the maximum number of shoots per explant (8.87 shoots). Especially, besides the appearance of shoots with green leaves, the surface of the leaves mostly was also enlarged, which implied that the enlarged leaf shoots were ready for the rooting phase (Figure 5).

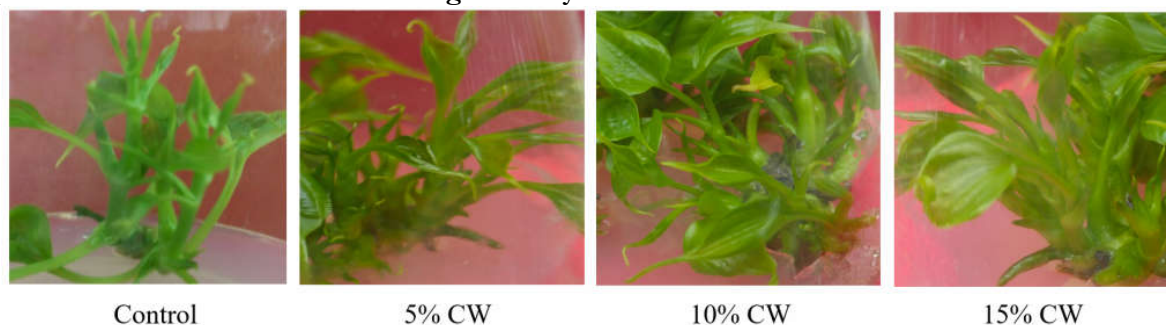


Figure 5. Effects of various concentrations of CW on *in vitro* shoot proliferation in *S. tuberosa*

Hence, enrichment of the culture medium with CW, which contains the natural growth regulators, sugar, some vitamins, acid amines, and enzymes, brought positive impacts on the useful shoot formation of *S. tuberosa*. The combined effect of BAP and CW has been reported in previous studies [8, 9] on the other crops which was in accordance with our results on the interaction of BAP and CW obtained on *S. tuberosa* in this study. 10% CW associated with the compatible concentration

of BAP (8.9 μ M) showed a synergistic effect producing a high rate of shoot multiplication in the Fig. of shoots in *Heliconia chartacea* "Sexy Pink" [9], while only 5% CW affixed to 3.0 mg/L BAP was suitable for the maximum Fig. of 6.3 multiple shoots in *Musa* cv. *Udhayam* [10].

4. CONCLUSION

Nodes of *S. tuberosa in vivo* were sterilized and regenerated shoots successfully

in vitro. The results of our study determined that the appropriate concentrations of BAP and sugar for shoot production in *S. tuberosa* were 1.5 mg/L and 30 g/L, respectively. Coconut water had a strong positive influence on *in vitro* shoot multiplication and the formation of effective shoots. MS medium supplemented with 1.5 mg/L BAP, 30 g/L sucrose and 10% CW was determined to be the optimal medium of shoot proliferation in *S. tuberosa* from the nodal explants, obtaining the maximum number of shoots per explant was 8.87 shoots.

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