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Low Cost Sterilization Technique and *In Vitro* Initiation of Vanilla (*Vanilla planifolia* Andr.)

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Abstract

Microbial contamination is one of the most serious problems of plant tissue culture. Treating the mother plant with chemicals and nourishing them in a greenhouse instead of letting them to laboratory directly from the field has greater importance to plant tissue culture techniques to reduce contamination los and to increase potency level. In the present study, effort was made to develop an effective surface sterilization and shoot imitation protocol with an enhanced survival rate of nodal explants of vanilla by treating with various sterilant combination with different exposure time and hormonal combinations to enhance initiation. The explants were collected from Jimma Agricultural Research Center greenhouse and used for the subsequent experiments. In all cases, the experiments were laid out in Completely Randomized Design (CRD) with factorial treatment combinations and replicated three times. In this study, statistically highly significant ($p<0.001$) differences were recorded from the use of different concentrations of active chlorine in local bleach (Berekina®) and varied levels of treatment durations on aseptic culture initiation. Therefore, the best result 82% rate of asepsis with survival rate of 80% was obtained from the use of 25 minutes treatment of nodal explants using a solution Berekina® containing 5% active chlorine. Regarding shoot initiation, the combined use of BA and Gibberellic Acid (GA3) revealed statistically very highly significant ($p<0.001$) differences, whereby 1.5 mg l^{-1} BA and 0.5 mg l^{-1} GA3 proved to be the best providing the mean length of 6.67 cm; node number 6 and mean leaf number 5 of shoot after five weeks of culture was initiated.

Keywords: Berekina®; Contaminants; Aseptic

Introduction

A good clean vanilla explant, once established in an aseptic condition, can be multiplied several times; hence, explant initiation in an aseptic condition should be regarded as a critical step in micropropagation [1]. Microbial contamination presents a major challenge to the initiation and maintenance of viable *in vitro* cultures. Contamination in this paper refers to the infection of vanilla explants by either fungus, bacteria, and/or yeasts [2], which are commonly found on the surfaces and in the natural openings of the explant material, and which become manifested during culture initiation and can either be overt or covert. Among several possible sources of contamination during *in vitro* culture, the explant material is one of the main sources of contamination and the explant surface carries a wide range of microbial contaminants, therefore it is imperative to use appropriate techniques to sterilize the explants prior to inoculation. Plant tissue culture media which are rich in sucrose and other organic nutrients readily support the growth of different microorganisms, including bacteria and fungi, which can grow relatively much faster than the cultured tissues on the medium, thereby killing the tissues at the end. Therefore, sterilization of explants is a key step in any plant tissue culture work, as the removal of all microorganisms is essential to attain successful initiation, growth and development of the cultured tissues *in vitro*, which otherwise would be overwhelmed by the contaminants [3-5].

On top of treating the explant material, tackling contamination during *in vitro* culture requires appropriate care to make sure all other possible sources of contamination are cleansed, as well. Among all these sources, those associated with the culture medium, culture vessels and the instruments that are used to handle explants, etc., could be tackled through autoclaving at 15 psi and 121°C for 15-20 minutes. Furthermore, the latter two could also be effectively cleansed through oven drying at 180°C for 3 hours. However, that source of contamination associated with the explant requires adoption of other control strategies. To reduce the level of microbial contamination of the stock plant and hence obtain axenic explants, various strategies have been and are still being employed in tissue culture labs. In this regard, the primary strategy is growing the stock plants under controlled conditions in the greenhouses, whereby they are supplied with proper nutrition, careful watering at their root base avoiding wetting of the foliage at any cost, together with their regular treatment with a contact and systemic antimicrobial chemical. The second option is stated as raising the stock plants in the field, but with regular treatment using contact and systemic antimicrobial agents prior to collection of the explants. The third strategy involves pruning of donor plants grown in the field so that they could produce new shoot tips that would have minimal microbial loads, which in turn could give contaminant-free explants [6]. Therefore, different workers had adopted the first strategy indicated above and used different types of explants from greenhouse grown healthy, well treated and actively growing stock plants of *V. planifolia*. Consequently, Geetha and Shetty and Kalimuthu et al. had used shoot tips and nodal explants for their micropropagation study of vanilla, while Janarthanam and Seshadri had used leaf segment and nodal explants. In line with this, there has been use of greenhouse raised vanilla stock plants as a source of nodal explants during their studies [7-9].

Among others, nodal explants are highly preferred in vanilla micropropagation, since they can provide sufficient quantities of elongated shoots having several nodal segments for successive sub culturing. Apart from this, nodal explants are suitable to have effective cultures for mass multiplication of *in vitro* shoots, rooting as well as ex vitro acclimatization, on top of its benefits to avert soma clonal variations [10]. Besides explants selection for plant tissue culture initiation, best initiation media formulation is very fundamental component for vanilla mass propagation through *in vitro* technique. Since there was no study done on vanilla initiation and initiation step of tissue culture can give good land mark for the consequent steps of plant tissue culture there is a need of optimizing proper initiation media for vanilla culture. In this study the explants selected was nodal, to break dormant buds and to enhance shoot initiation and development, proper media formulation at the start of tissue culture is crucial.

Regarding establishment of axenic cultures primary consideration is given to the selection of suitable sterilizing agents and determination of appropriate duration of explant exposure to the chemicals. As a result, the chemical selected should be of a type that could be easily removed from the explant surface through repeated rinsing [11], while the duration of exposure is sufficient enough to ensure higher rates of explant survival after treatment. However, as the use of some antimicrobial chemicals (such as antibiotics, and/or some carcinogenic chemicals like mercuric chloride) could possibly harm the users, on top of causing phytotoxicity, retarded explants growth, as well as encouraging resistance buildup for the chemicals, it is essential to take the utmost care while selecting the chemical for use. Hence the purpose of this study was to determine the optimum concentration of local bleach (Berekina®) solution and duration of exposure of vanilla nodal explants for successful sterilization and to optimize the combination of BA and GA3 concentration for best vanilla *in vitro* initiation (Figure 1).

Materials and Methods

Experimental layout

Effect of sterilant chemical concentration and exposure time on the contamination rates of vanilla explants (Experiment I): Young and actively growing stem nodes from the greenhouse-maintained stock plants of the *Vanilla planifolia* clones were collected, and thoroughly washed with liquid soap (Largo®) and tap water. Then after, they were left under running tap water for 30 minutes to reduce load of contaminants on their surface, as well as to expose the hidden microbes within perforations of the explants. Subsequently, the nodal explants were reduced to two nodes each with hanging internodes at both sides and soaked in a solution of 3 g 1^{-1} Kocide-101 for 30 min. After 4 times rinsing with sterilized distilled water, the explants were transferred to the laminar air flow hood and dipped in a 70% ethanol solution for one minute, prior to their exposure to the different sterilization treatments. During sterilization, the explants were subjected to different treatments having varied concentrations (i.e., active chlorine percentage of 1%, 3% or 5%) of the commonly available surface sterilization chemical, local bleach (Berekina®), for 10, 15, 20 or 25 minutes duration under aseptic condition. In this experiment, following the recommendations of Padhi and Singh and Tilahun et al, a 0.1% Mercury chloride solution with 10 minutes exposure time was used as a control. In all cases, the culture vessels containing the explants with the sterilizing agent were gently shaken [12,13].

The experiment was laid in a 3 × 4 factorial treatment combinations in CRD, i.e., three levels of chemical concentrations (i.e., 1%, 3% and 5% active chlorine (v/v) in a local bleach and four levels of exposure time (10, 15, 20 and 25 minutes) with three replications. To enhance the efficacy of the sterilant chemical, two drops of Tween-20 (as a wetting agent) was added into each treatment. Finally, in all cases, the explants were thoroughly rinsed (4 times) with sterilized distilled water in a sterilized beaker to get rid of the chemical residues on the explant surface.

Explant culture: Inside the laminar flow hood, the surface sterilized explants were laid on sterile Petri dishes and further excised with the help of sterile forceps and blade to remove the chlorine affected tissues at both ends. In due time, their sizes were reduced to a single node with internodes at both sides. Then after, the trimmed explants were cultured in 370 cm^3 volume Manson jars containing 40 ml of the conditioning medium, i.e., a PGR-free MS basal medium. Finally, vanilla nodal explants of approximately 2 cm length were inoculated on to the conditioning medium. After culturing, the mouth of each jar was properly closed with its cap and sealed with a strip of parafilm. The jars were then labelled, indicating the media code, culturing date, and name of the clone before they were randomly placed on the Dixon shelves within the growth room. After six days culture on the conditioning medium, non-contaminated and surviving cultures were recorded and transferred to the shoot initiation experiment media.

Experiment II: The effects of combinations and concentrations of Benzyl Adenine (BA) and Gibberellic Acid (GA3) on vanilla *in vitro* shoots initiation: In this experiment, MS basal medium supplemented with 30 g l^{-1} sucrose with four levels of BA (0.5, 1.0, and 1.5 mg l^{-1}) in combination with three levels of GA3 (0.0, 0.5, and 1.0 mg l^{-1}). The PH of the medium was adjusted to 5.75 before it autoclaved and gelled with 1% agar. Therefore, the experiment was laid in a 3 × 3 factorial combination in CRD, whereby the two factors being BA and GA3 each at three treatment levels with three replications and four explants per treatment. 25% L-cystine and 100 g myo-inositol were added as additives. Shoots initiated at this media were sub-cultured onto a PGR-free medium for at least two weeks prior to their use in subsequent experiments, to remove any carry-over effect of the plant growth regulators that were used at the previous culture stages. Unless stated otherwise, in all cases the culture jars containing the explants were placed randomly in the growth room having an average of 50-60% relative humidity, temperature of 24-26°C and 2500 lux of light intensity with 16 hr duration of light [14].

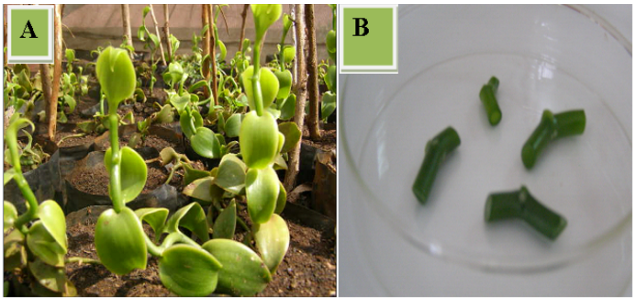

Figure 1: Vanilla stalk plant and explants preparation for shoot initiation; (A) Vanilla mother plant used for explant source in the green house (B) Aseptically prepared vanilla explants in biosafety laminar flow hood cabinet for shoot initiation experiment.

Varied data were collected from the two experiments during the study. These include:

Percentage of contamination: It is a quantitative character evaluated after the sterilization treatment, to determine the effectiveness of the sterilizing chemical concentrations at various durations of explant exposure. These were obtained by counting number of cultures contaminated divided by the total number of explants treated with the same treatment and multiplied by 100 after three weeks of culture.

Types of infection: It is a qualitative character considered in the sterilization experiment. It was obtained by counting the type of contaminants divided by total contaminated explants and multiplied by 100.

Percentage of survival: It is a quantitative character considered in the sterilization experiment. This data shows the effectiveness of the sterilizing chemical and duration of exposure without being lethal. It was obtained through counting the surviving explants tolerating the phytotoxicity divided by the total number of sterilized explants in the same treatment and multiplied by 100 after three weeks of culture.

Average length of green shoot initiated: It is a quantitative character considered in shoot initiation experiment. The data were obtained through measuring the length of shoots in cm from the stem base to the shoot tip after five weeks of culture.

Number of leaves: It is a quantitative character considered in shoot initiation experiment and data were obtained through counting the number of leaves developed from a single explant and divided by the number of shoots produced from the explant within four weeks of culture.

Number of nodes: It is a quantitative character considered in shoot initiation experiment and data were obtained through counting the number of nodes developed from a single explant and divided by the number of shoots produced from the explant within five weeks of culture.

Statistical analysis

The collected data were analyzed using the SAS statistical software (Version 9.2) and mean separation were made following the procedure of REGWQ (Ryan-Einot-Gabriel-Welsch Multiple Range Test). Linear correlation was applied for sterilant and contamination.

Results and Discussion

Experiment I: The effect of sterilant chemical concentrations and exposure time on disinfection of vanilla explants

In this study, the differences in the rate of explant contamination due to the combined effects of both concentrations of the sterilizing agent and durations of exposure to the sterilant chemicals were highly significant ($p<0.001$) (Table 1). The highest loss of cultured explants (99.33% and 96.70) due to microbial contamination was recorded when vanilla explants were treated for 10 and 15 minutes respectively with the local bleach (Berekina®) solution containing 1% active chlorine (Tables 1 and 2).

Source of variation	Mean squares	
	df	Cont.
Chem. conc	3	8492.81***
Exposure time	3	2339.96***
Chem. conc* exposure time	6	543.29***

Table 1: Mean square values for the effect of different concentrations of sterilizing chemicals (Berekina®) and treatment exposure time on contamination rates of vanilla explants. Chem. conc=Chemical concentration, df=Degree of Freedom, Cont.=Contamination, ***=P<0.001.

A general reduction trend in contamination level of vanilla nodal explants (from 99.33% to 18%) was observed with increasing concentrations of the local bleach solution from 1% to 5% active chlorine together with a concomitant increase in exposure time from 10 to 25 minutes (Table 2). In this experiment the highest the highest contamination free (82%) with survival rate 80% of explants was observed from a treatment that involved a 25 minutes sterilization of nodal explants with 5% active chlorine Berekina® solution, followed by a 20 minutes treatment of explants with a similar level of active chlorine concentration. In most cases, local bleach (Berekina®) did not exert any adverse effect on the explants, thus the surviving explants from the respective treatments were completely green and actively growing on the initiation media. This could be ascribed to the comparatively lower toxicity of the sterilant chemical, local bleach or Berekina®.

During this experiment, 2% of explant death was recorded from those combinations involving longer exposure time (25 minute) and the highest level of chlorine concentration (5%) in the sterilant chemical (Data not shown). In this study, nodal explants treated with 5% active chlorine concentration for 25 minutes exposure time showed an equivalent aseptic rate to those subjected to the positive control (10 minutes treatment with a 0.5% Mercury chloride solution) (Table 2). However higher survival rate of explants was recorded the one which were treated using 5% active chlorine local bleach for 25-minute exposure time compared to the positive control. In the current study, contamination level of vanilla nodal explants revealed a very highly significant ($p<0.001$) and inversely associated with the level of active chlorine concentrations within the local bleach (Berekina®)

In this experiment, fungal and bacterial contaminants were easily detected by the naked eye, as they produced visible symptoms on the culture medium. Therefore, fungal contaminations were characterized

by their "fuzzy" appearance of multitude of colors that are associated with their mycelial growths [15]. In summary, the major source of contamination in the present study was observed to be of fungal origin (95%), unlike the relatively rare appearance of those associated with bacteria (5%). Fungal contaminants were grayish green and white in color (Figures 2A and 2B), whereas bacterial contaminants produced mucoid structures with patterns on the culture media (Figure 2C). In all cases, aggressive and overwhelming growth of the microbes was observed on the culture medium, as compared to that of the explants, whenever the treatments involved lower rates of active chlorine concentrations and/or shorter exposure durations of the explants to the sterilant chemicals, and vice versa.

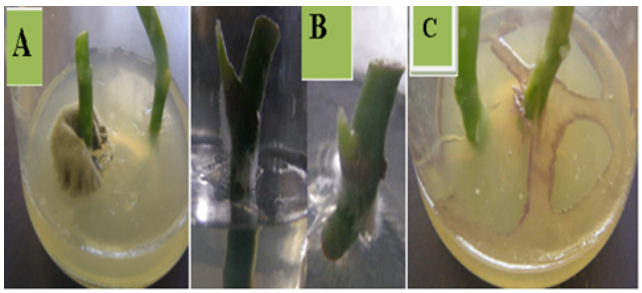

Figure 2: Contamination types observed on *in vitro* culture initiation of vanilla explants; (A) and (B) Different colors of fungal contaminants at different stages of vanilla culture initiation (C) Bacterial contaminants on cultured vanilla explants.

Treatment	% Active Chlorine Concentration (V/V)	Exposure Time (Minutes)	Level of Contamination (%)
Control (Mercuric Chloride)	0.5	10	16.67 ± 1.5^{h}
Local Bleach (Berekina®) Solution	1	10	99.33 ± 1.5^{a}
	1	15	96.70 ± 1.55^{a}
	1	20	91.33 ± 1.5^{b}
	1	25	87.33 ± 1.54^{cd}
	3	10	90.66 ± 1.54^{bc}
	3	15	86.66 ± 1.55^{d}
	3	20	82.66 ± 1.5^{e}
	3	25	57.33 ± 2.3^{f}
	5	10	85.33 ± 1.4^{de}
	5	15	58.66 ± 2.3^{f}
	5	20	32.00 ± 2.0^{g}
	5	25	18.00 ± 2.0^{h}
CV(%)			2.21

Table 2: Effect of sterilant chemical concentrations and durations of treatment on the contamination rates of vanilla nodal explants after 3 weeks of *in vitro* culture.

Treatment means within a column followed by the same letter are not significantly different from each other using REGWQ at $P<0.05$.

The higher prevalence of fungal contamination in those treatments involving lower rates of active chlorine and/or shorter durations of exposure could be attributed to the poor performance of the sterilant chemical at lower concentrations and/or insufficiency of the durations for which the explants were exposed to the chemicals to bring the desired effect. As stated by Shafique et al., it could also be associated with the relatively higher rates of multiplication and mycelial growth of the fungal microflora under the prevailing favorable acidic (pH 5.75) culture medium conditions, which favored more the latter's growth [16]. Determination of optimal levels of chemical concentrations and durations of explant exposure to the sterilant is highly important to ensure effective control of associated microorganisms and establishment of aseptic culture, without compromising explant survival. The present study had thus confirmed the possibility of getting up to (82%) contaminant-free explants, making use of a 25 minutes treatment of nodal pieces of *V. planifolia* using the local bleach Berekina® solution containing 5% active chlorine (Table 2).

The efficacy of Berekina® on sterilization of vanilla nodal explants in the current study seems a promising alternative to the previous recommendation of Zerihun et al., who only succeeded from using a 5 minutes treatment with 0.1% solution of the hazardous mercuric chloride ($HgCl_2$) solution. The rate of survival during aseptic culture initiation (80%) recorded from this study was better than the one obtained by the above-mentioned workers (60% survivable rate). On top of these, the use of Berekina® is by far better than that of mercuric chloride, which is strongly discouraged due to its extreme toxicity to plants and animals; beside its costliness, which only make its recommendations so exceptional, i.e., only when other techniques proved ineffective [17,18]. On the other hand, the broad-spectrum efficacy of bleach is particularly ascribed to its peculiar reactivity with microbial cells, i.e., its potentials to react so quickly and denature the cells of most microbial contaminants [19]. Therefore, use of the locally produced, readily available and cheaper Berekina® solution, containing 5% active chlorine for 25 minutes was also preferred to use the commercial mercury chloride ($HgCl_2$) solution, which is an imported and costly product.

Experiment II: The effects of combinations and concentrations of Benzyl Adenine (BA) and Gibberellic Acid (GA3) on vanilla *in vitro* shoots initiation

In the present study, the combined use of BA and GA3 had significantly supported vanilla *in vitro* initiation. Therefore, the two-way interaction of these two phyto-hormones had revealed highly significant differences ($p<0.001$) regarding mean shoots length, leaves and nodes development of vanilla *in vitro* after five weeks of culture (Table 3).

Source of Variation	Mean squares			
	df	Shoot Length	Node number	Leaf Number
BA	2	0.9292^{***}	1.03^{***}	0.11^{ns}
GA3	2	24.38^{***}	13.37^{***}	16.44^{***}

BA × GA3	4	1.67***	1.87***	1.05***

Table 3: Mean square values for The Effects of Combinations and Concentrations of Benzyl Adenine (BA) and Gibbrelic Acid (GA3) on Vanilla *in vitro* Shoots Initiation ***=P<0.001 BA=6-benzyl Adenine, GA3=Gibberellic Acid.

Accordingly, the maximum mean length of shoots were produced during *in vitro* initiation of vanilla (6.67 cm) (Figure 3C) and maximum number of leaves and nodes which was 5 and 6 respectively were recorded from those explants of vanilla cultured on MS basal medium added with 1.5 mg l^{-1} BA and 0.5 mg l^{-1} GA3, while the minimum average length of shoot (3.16, 3.23 cm and 3.56 cm) and the least leaf and node number (2 and 3 respectively) were obtained from the culture medium supplemented with 0.5, 1 and 1.5 mg l^{-1} BA only (Table 4).

In this study the phytohormone combinations of 0.5 mg l^{-1} BA with 1 mg l^{-1} GA3 was insignificance in vanilla shoot length and leaf number with the treatment of 1.5 mg 1^{-1} with 0.5 mg l^{-1} GA3, however significantly different in node number, hence the latter media combination found to be best to produce elongated vanilla shoot which could be suitable for mass multiplication programs. On the contrary media supplemented with solely BA in the absence of GA3 were less effective in bringing elongated shoot development and other shoot parameters rather stunted growth was observed. Hence the presence of GA3 hormone found to be pertinent for vanilla nodal shoot initiation. More over when the media is fortified solely with GA3 increased pattern also showed elongated shoot however when we look at the shoots get thinner and thinner when the amount of GA3.

Treatments				
BA (mg/l)	**GA3(mg/l)**	**Shoot Length(cm)**	**Leaf Number**	**Nod number**
0.5	0	3.56 ± 0.15^{d}	2.66 ± 0.57^{c}	3.00 ± 0.0^{cd}
0.5	0.5	4.63 ± 0.35^{c}	4.00 ± 0.00^{b}	4.00 ± 0.0^{bc}
0.5	1	6.93 ± 0.11^{a}	5.00 ± 0.57^{a}	4.00 ± 0.0^{bc}
1	0	3.23 ± 0.20^{d}	2.00 ± 0.0^{c}	3.00 ± 0.0^{cd}
1	0.5	5.66 ± 0.15^{b}	4.33 ± 0.5^{7}	4.66 ± 0.0^{b}
1	1	5.83 ± 0.58^{b}	5.00 ± 0.0^{ab}	4.33 ± 0.57^{b}
1.5	0	3.16 ± 0.15^{d}	2.00 ± 0.0^{c}	2.33 ± 0.0^{d}
1.5	0.5	6.67 ± 0.11^{a}	5.00 ± 0.0^{a}	6.00 ± 0.0^{a}
1.5	1	6.73 ± 0.25^{a}	4.00 ± 0.0^{b}	4.33 ± 0.0^{b}
CV (%)		5.28	7.2	4.76

Table 4: The effects of combinations and concentrations of benzyl adenine (BA) and gibberellic acid (GA3) on vanilla *in vitro* shoot initiation.

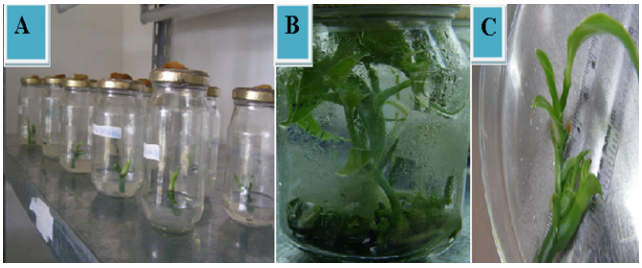

Figure 3: Vanilla *in vitro* culture initiation: A. Nodal explant bud dormancy breakage and shoot initiation during *in vitro* culture B. Initiated and well elongated shoots which are potential for multiplication experiment C. Elongated vanilla shoot after five weeks of culture ready to data collection of some growth parameters.

Conclusion

During micropropagation, it is essential to make use of all the steps that are indispensable to mass propagate *V. planifolia*. The first of these is sterilization of selected explants, which is a key step for removing any sorts of microbial contaminants from the explant surface; thereby attain successful *in vitro* establishment that is the basis to subsequent growth and development of the tissues. During this study, a 25 minutes treatment of the nodal explants with a 5% active chlorine solution of locally available bleach, Berekina®, had successfully cleansed the explants resulting in 82% contaminant-free explants established *in vitro*.

The sterilization technique developed in the present study would be so beneficial to the spice sub-sector since it had come up with a suitable technique for effective establishment of vanilla nodal explants under *in vitro* condition. The present finding could also be of paramount importance as it avoids the need for using the imported, hazardous and costly mercuric chloride solution for cleansing explants. Instead, it had devised effective ways for using the locally available bleach, Berekina®, at a rate of 5% active chlorine for 25 minutes duration. Initiation media formulation is so curtailed, and which could determine the success of plant tissue culture programs. Unless the arrested explants ignited at the start of the *in vitro* culturing system it could be impossible to achieve the planned objective of plant tissue culture. Once proper start of plant growth and propagation is induced it could be good resource for the next activities. Hence in the study a very potential protocol was developed to initiate shoots from vanilla nodal explants.

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