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Conservation of Vanilla Planifolia by In-Vitro Micropropagation Method

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Abstract- Vanilla planifolia is a tropical monopodial orchid, commercial spice crop known for its popular flavoring substance called vanillin. Traditionally, Vanilla is propagated by stem cutting which is labour intensive and time consuming process. As Vanilla is a monopodial orchid, its growth is hampered once the apical tip is lost. A simple and efficient micropropagation protocol was developed for conservation of this plant using nodal segments as explants. The explants were cultured on Murashige and Skoog (MS) basal medium supplemented with varying combinations of N6- benzyladenine, Kinetin and Casein hydrolysate. Among different concentrations, MS supplemented with N6-benzyladenine (1mg/l), Kinetin (0.5mg/l) and Casein hydrolysate (500mg/l) produced more number of better sized shoots. These plantlets were hardened successfully and transferred to pots.

Key words: Vanilla planifolia, nodal explant, plantlets.
Abbrevations: BAP- benzyl amino purine; KN- kinetin; CH- Casein hydrolysate; MS - Murashige and Skoog's media.
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1. INTRODUCTION

Vanilla planifolia is an herbaceous, perennial, climbing, terrestrial, monopodial orchid belonging to family Orchidales. It is commercially cultivated for pods from which popular flavouring substance called vanillin is extracted. Vanillin is used in flavouring ice creams, soft drinks, condiments, in cosmetics and perfumery industry. Due to this it is second most expensive spice in the world market, next to saffron.

Vegetative propagation of plants has been practiced for centuries and many improvements in conventional methods have been introduced over the years. Applications of the plant tissue culture have expanded both, the scope and potential of vegetative propagation. *Vanilla* is propagated by stem cuttings. However, this method of propagation is not economical and is time consuming. Loss of apical tip arrests the growth and development of the mother plant. It also does not fully meet with the market demand for propagules. In order to conserve the plant and meet the demand for propagules, the rapid regeneration of this species is essential. Also *Vanilla* is usually infected by viruses such as Cymbidium Mosaic Virus (CMV), Udontoglosum Ring Spot Virus and Vanilla Mosaic Virus.

Tissue culture techniques have definite and indispensible advantages over the former, as it ensures an extremely rapid rate of multiplication with conservation of the species. It is not season dependent and requires only a limited quantity of plant tissue as a source of initial explants. Micropropagation of *Vanilla planifolia* has been reported through callus culture, protocorms, root tips and axillary bud explants. The technique has great potential for rapid and large scale multiplication of true to type planting material (Pierik, 1990). This process is less time consuming and removal of small amount of appropriate tissue without harming the *in situ* population is a convenient technique (Carlos Alberto Cruz – Cruz, Meria Teresa Gonzalez – Arno and Florent Engelmann, 2013). The primary gene pool of *V. planifolia* is narrow and is evidently threatened due to destruction of its natural habitats making the secondary gene pool important as a source of desirable traits especially for resistance to diseases. Tissue culture techniques ensure production of virus free propagules.

First report on organogenesis in Vanilla was by Zhuping *et.al* in (1987), where plantlets were produced via callus. Philip and Paul (1990) have shown that, meristem cultures were an ideal source for long term storage of pathogen free germplasm through cryopreservation. Efficient micropropagation was carried out by P. S. George and G. A. Ravishankar (1997) followed by S. Geetha and Sudheer Shetty (2000).

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2. MATERIAL AND METHODS

Preparation of explant:

Apical buds were excised from Vanilla mother plant collected from Phulsange Nursery in Parandawadi, Talegaon Dabhade. The explant was pre-treated with labolene solution followed by 5% Bavistin solution for 20 min. The buds were washed several times with tap water and then were treated with 0.1% Savlon and then washed with distilled water for

several times. Surface disinfestations were effected with 70% ethanol and 0.1% mercuric chloride ($HgCl_2$) for 7min followed by 4-5 rinses in sterile distilled water. The working table of laminar airflow cabinet and spirit lamp were sterilized by swabbing with absolute alcohol. All the required materials like media, spirit lamp, matchbox, glassware etc., were transferred on to the clean laminar airflow. The UV light was switched on for half an hour to achieve aseptic conditions.

Inoculation:

The explants were taken on glass plate and the bleached parts of the explants were cut off or removed using sterile blade. Then the explants were inoculated on MS medium supplemented with 3% sucrose and varying concentrations of KN and BAP (ranging from 0.0 to 5.0mg/l). Then the plantlets were transferred to medium containing CH (ranging from 0.0 to 1000mg/l), KN (0.5mg/l) and BAP (1mg/l). The culture bottles were then sealed using clean wrap or plastic wrap. Then the bottles were kept in incubated at 26° C ± 2°C under a 12hr photoperiod with 6mE/m^2/s light intensity.Subculturing was done after every 25-30 days. For rooting, 3-4cm long shoots were kept on MS full, MS half and MS ¼ medium which was supplemented with 2% sucrose and kept under specific physical conditions (Table III). In order to enhance rooting from in-vitro regenerated shoots, the effect of IAA, IBA and NAA (varying in concentrations from 0.0-5.0mg/l) supplemented into MS ½ medium were tested. The auxin mixture was tested at four different concentrations, each auxin added at a concentration range from 0.0-5.0mg/l in MS ½ medium supplemented with 2% sucrose. After inoculation of excised shoots, incubation was carried out in light at 26±2^{0}C with 6µE/m^2/s light intensity under 16hr photoperiod.To test the effect of charcoal, MS full, MS half and MS ¼ medium supplemented with 2% sucrose was used with 0.25% activated charcoal. The treatment was given both in light and dark conditions.

Regenerated shoots with well developed roots were transferred to plastic pots containing potting mixture with varying substrates (Table VII). The pots were kept inside the shade house with relative humidity 50-60% for 20-25 days. Relative humidity was decreased gradually and the plantlets were brought out of the tunnel exposed to natural environmental conditions.In order to enhance rooting from in-vitro regenerated shoots, the effect of IAA, IBA and NAA (varying in concentrations from 0.0-5.0mg/l) supplemented into MS ½ medium were tested. The auxin mixture was tested at four different concentrations, each auxin added at a concentration range from 0.0-5.0mg/l in MS ½ medium supplemented with 2% sucrose. After inoculation of excised shoots, incubation was carried out in light at 26±2^{0}C with 6µE/m^2/s light intensity under 16hr photoperiod.

3. RESULT

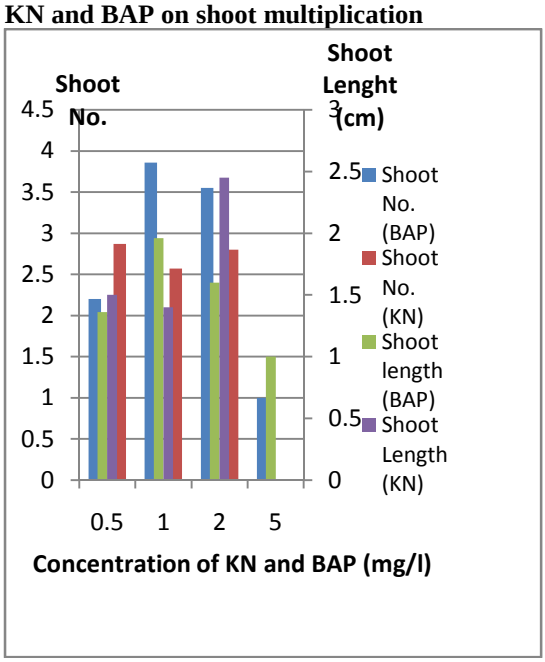
In the present experiment, MS supplemented with 3% sucrose was used as the basal medium. Cultures were maintained at 26±2^{0}C, under 16h photoperiod with 6µE/m2/s light intensity provided by cool white fluorescent light.

Differential response was obtained by using different levels of BAP combined with Kin and CH. Significant difference was obtained among treatments for multiple shoot initiation for each explant. After the first subculture, an average no. of 2-3 shoots that can easily be separated and cultured on fresh media were produced for the above mentioned hormone combinations.

Effect of different combination of KN and BAP:

For shoot multiplication, different combinations of KN and BAP ranging from 0.5 to 5.0mg/l were added to MS medium supplemented with 3% sucrose. It was observed that at 0.5mg/l KN and 1mg/l BAP, maximum number of shoots (4.4±0.4) were obtained with shoot length increased to 2.4 ± 0.3cm. As the KN concentration increased, the number of shoots decreased (Table I). (Fig1)

Fig:1 Effect of different concentrations of KN and BAP on shoot multiplication



The effect of BAP was tested at different concentrations by incorporating it into MS medium supplemented with Kinetin, CH and 3% sucrose. It was observed that at 1mg/l BAP, 4.4±0.4 shoots were obtained with shoot length of 2.4±0.3cm. No shoot multiplication was observed in absence of BAP. Also higher concentrations of BAP were inhibitory for shoot multiplication.

The effect of KN was tested by incorporating it, at varying concentrations ranging from 0.0 to 5.0mg/l into MS medium supplemented with BAP, CH and 3% sucrose. With incorporation of KN (0.5mg/l), maximum number of shoots was obtained. The shoot length at 0.5mg/l KN was 2.4 ± 0.3cm and maximum 4.4±0.4 shoots were obtained. No shoot multiplication was observed in absence of KN. Also with increasing KN concentrations in the medium, the no. of shoots declined.

In the present study, CH at a concentration of 500mg/l the no. of regenerated shoots increased to 6-7 per explant and shoot length was 2.9±0.3cm. In absence of CH, no shoot multiplication was observed. With increasing concentration of CH there was a decrease in the number and length of shoots (Table II).

Effect of different auxins:

All auxins induced rooting, the number of roots and the length of roots however varied with the auxin tested. With increase in IBA and IAA concentrations from 0.5-3.0mg/l rooting was 100%, but time required for root initiation was more than that observed in auxin free media. Further increase in concentration decreased rooting upto 60% to 70%. As concentration of NAA was increased upto 3.0mg/l, number of roots and root length decreased with 60% rooting and rooting initiation was seen after 50 days.

The optimum concentration of auxin mixture is 1.0mg/l. When 1.0mg/l auxin mixture was added, almost 4-5 roots were produced with root length 3.5±0.3cm, with 100% rooting. Rooting initiation was observed after 33 days. As the concentration increased rooting percentage decreased.

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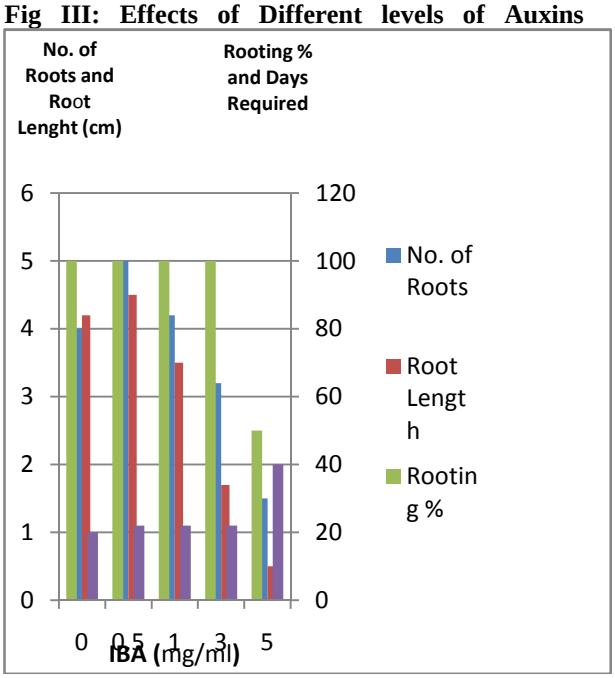
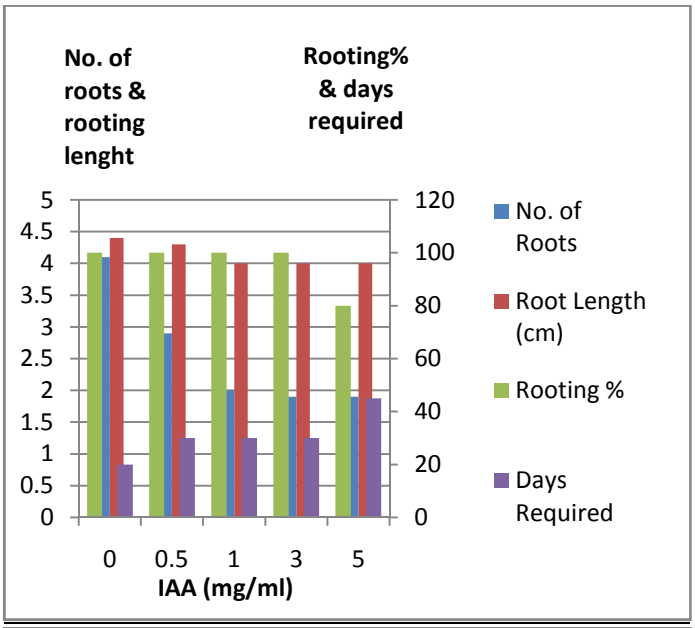
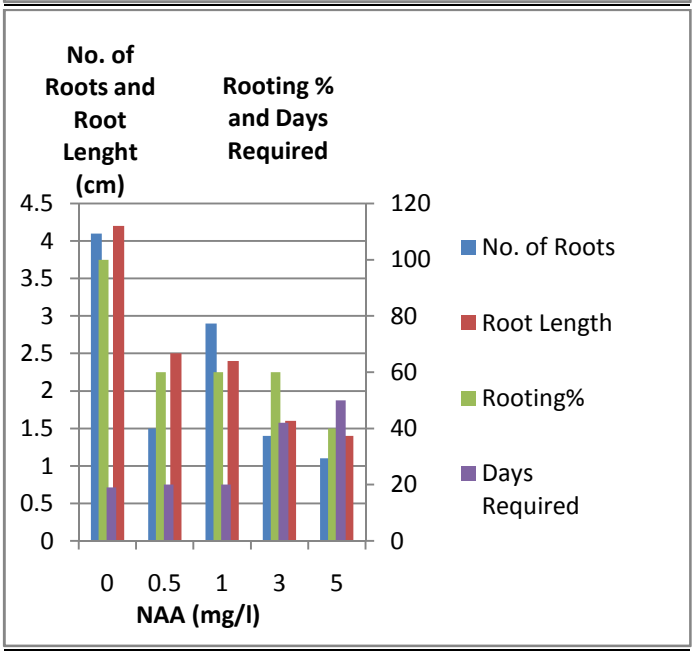
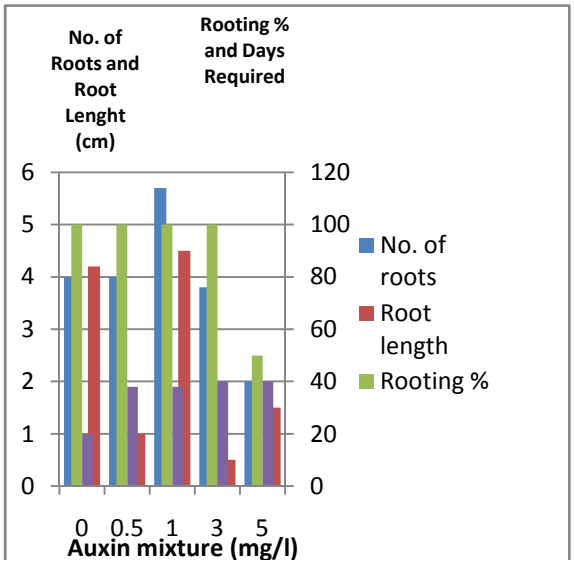
Fig III: Effects of Different levels of Auxins









Effect of Low Salt Medium on rooting:

\Different strengths of basal media like full, half and one fourth with 3% sucrose were tried for rooting experiments. These were carried out in light, dark and light and dark alternatively. Shoots inoculated on MS full strength medium initiated 3.4±0.2 roots within 27 days. 75% rooting was obtained with root length being 4.2±0.4cm. In dark, the rooting percentage decreased to 50% and the days required for rooting initiation were 37 days with root length 3.6±0.3cm. In alternate light and dark conditions, rooting percentage decreased to 35%.

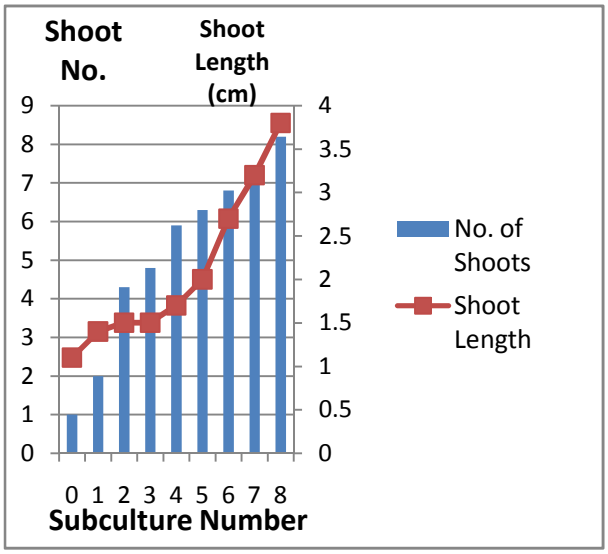
Shoots inoculated on MS half strength medium initiated 4.1±0.1 roots within 22 days in light. 100% rooting was obtained with root length being 3.6±0.1cm. In dark, the rooting percentage decreased to 60% and the days required for rooting initiation were 32 days with root length 5.1±0.1cm. In alternate light and dark conditions, rooting percentage decreased to 50% and root length was 4.6±0.3.

Shoots inoculated on MS one fourth strength medium and kept in light, initiated 2.1±0.2 roots within 37 days. 40% rooting was obtained with root length being 2.5±0.3cm. In dark, the root length decreased to 1.7 cm with rooting percentage being 30% and the days required for rooting initiation were 32 days. In alternate light and dark conditions, rooting percentage decreased to 28% and root length was 2±0.2cm.

Effect of Subculture:

Subcultures were carried out after every 40-45 days interval. The nodal explants inoculated were 1.0±0.5cm. The no. of shoots produced per culture gradually increased from 2.0±0.1 to 8±0.1shoots from first subculture to 8th subculture. A gradual increase in the shoot length was observed as the subculture number progressed (Table V).

Fig II: Effect of Subculture:



Effect of different substrates on survival of shoots:

In present study, different combination were tried to improve survival of plants. The rooted plants were transferred to soil in different combinations of sand, soil, brick pieces and other substrates. The best results obtained were with sand: soil: brick pieces (4:1:1), 80% shoots surviving. The shoots were green and healthy. Plants transferred to soil could not tolerate exposure to direct sunlight but survived in shaded environment (Table VII).

Table I: Effect of various combinations of KN and BAP on shoot multiplication and shoot length

Basal Media: MS+3% Sucrose
Temperature: 26 ±2^{0}C
Light intensity: 6µE/m^2/s
Photoperiod: 16hr light
Supplements: KN and BAP

KN (mg/l)	BAP (mg/l)	No. of Shoo	Callu	Shoot Lengt (cm)
0.5	0.5	2.1±0.4	-	1.3±0.3
0.5	1.0	4.4±0.4	-	2.4±0.3
0.5	2.0	4.0±0.2	-	1.4±0.5
0.5	5.0	1.0±0.0	-	1.0±0.0
1.0	0.5	2.2±0.3	-	1.3±0.2
1.0	1.0	4.0±0.3	-	1.6±0.2
1.0	2.0	3.1±0.0	-	1.8±0.4
1.0	5.0	1.0±0.0	-	1.0±0.0
2.0	0.5	2.4±0.5	-	1.5±0.3
2.0	1.0	3.2±0.2	-	1.9±0.4

Table II: Effect of CH concentrations on Shoot Multiplication

Basal Media: MS+3% Sucrose
Temperature: 26 ±2^{0}C
Light intensity: 6µE/m^2/s
Photoperiod: 16hr light
Supplements: KN (0.5mg/l) + BAP (1mg/l)

Concentration of CH (mg/l)	No. of Shoots	Shoot Length (cm)
0	2.0±0.0	1.3±0.4
100	3.1±0.4	1.6±0.2
500	6.6±0.4	2.9±0.3
1000	3.7±0.3	1.8±0.3

Table III: Effect of Low Salt Medium on Rooting

Temperature: 26±2^{0}C
Light intensity: a) 6µE/m^2/s b) Nil
Photoperiod: a) 16h light b) Dark
Supplement: 2% Sucrose

Medium	Treatment	No. of Roots	Root Length (cm)	Rooting %	±2days required for root initiation
MS	Light (a)	3.4±0.2	4.2±0.4	75	27
	Dark(b)	2.9±0.3	3.6±0.3	50	37
	Alternately in light and dark	1.7±0.2	3.0±0.3	35	37
MS 1/2	Light (a)	4.1±0.1	3.6±0.1	100	22
	Dark (b)	3.6±0.3	5.1±0.1	60	32
	Alternately in light and dark	2.9±0.3	4.6±0.3	50	32
MS 1/4	Light (a)	2.1±0.2	2.5±0.3	40	37
	Dark(b)	1.7±0.2	2.0±0.1	30	42
	Alternately in light and dark	1.5±0.25	2.0±0.2	28	42

Table IV: Effect of different combinations of auxins and cytokinins on growth and shoot multiplication:

Basal Media: MS+3% Sucrose
Temperature: 26 ±2^{0}C
Light intensity: 6µE/m^2/s
Photoperiod: 16hr light
Supplements: Auxins + Cytokinins

Auxins (mg/l)		Cytokinins (mg/l)		Morphogenetic Responses and Frequency		
		KN	BAP	Shoots	Callus	Roots
2, 4- D	0.5	0.5	-	-	+	-
	2.0	0.5	-	-	++	-
	0.5	-	1.0	1	-	-
	2.0	-	1.0	1	-	-
	0.5	0.5	1.0	1	++	-
	2.0	0.5	1.0	1	++	-
IAA	0.5	0.5	-	-	-	-
	2.0	0.5	-	-	-	-
	0.5	-	1.0	-	-	-
	2.0	-	1.0	-	-	-
	0.5	0.5	1.0	-	-	1
	2.0	0.5	1.0	-	-	1
NAA	0.5	0.5	-	-	-	-
	2.0	0.5	-	-	-	-
	0.5	-	1.0	-	-	-
	2.0	-	1.0	-	-	-
	0.5	0.5	1.0	-	-	1
	2.0	0.5	1.0	-	-	1
IBA	0.5	0.5	-	-	-	-
	2.0	0.5	-	-	-	-
	0.5	-	1.0	-	-	-
	2.0	-	1.0	-	-	-
	0.5	0.5	1.0	-	-	1
	2.0	0.5	1.0	-	-	1

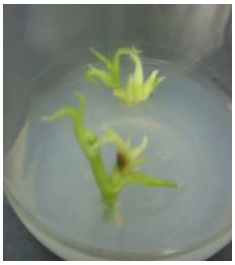
- = No Response
\+ = 100-400mg/tube callus
++ = 400-800 mg/tube callus

Table V: Effect of Subculture:

No. of Subculture	No. of Shoots	Shoot Length (cm)
0	1	1.1
1	2	1.4
2	4.3	1.5
3	4.8	1.5
4	5.9	1.7
5	6.3	2.0
6	6.8	2.7
7	7.1	3.2
8	8.2	3.8

Table VI: Effect of different substrates on survival of shoots.
Temperature: 26 ± 2^0C
Light intensity: 6µE/m^2/s
Photoperiod: 16h light
Relative humidity: 50-60%
Supplements: Different substrates

Substrate	Types of shoots	Survival %	Survival weeks
Autoclaved soil	Rooted	15	1-2
	Unrooted	5	<1
Sand	Rooted	20	2-3
	Unrooted	5	1
Soil	Rooted	10	1-2
	Unrooted	10	1
Sand: soil(1:1)	Rooted	50	4-5
	Unrooted	10	1
Sand: soil: brick pieces(4:1:1)	Rooted	80	>80
	Unrooted	15	1




Day 1 inoculation

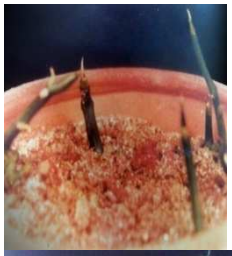
axillary bud sprout after 40 days


Shoot elonation for rooting

multiple shoots on nodes


Multiplication of shoots after 3rd subculture (5-7 shoots)

In vitro Rooting on MS half strength




Shoots trandered to soil (2 months old)

Plantlet after 12 months

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