International Journal of Herbal Medicine
Available online at www.florajournal.com

I J H M International Journal of Herbal Medicine

ISSN 2321-2187
IJHM 2014; 2 (4): 50-56
Received: 20-12-2014
Accepted: 17-01-2015

Aravind Barad
Biosystematics and Medicinal Plants Laboratory, Department of Botany, Gulbarga University, Gulbarga -585 106, India.

Vijaykumar B. Malashetty
Department of Safety Assessment, Advinus Therapeutics Limited, Bangalore-560058, India.

Seetharam Y. N.
Biosystematics and Medicinal Plants Laboratory, Department of Botany, Gulbarga University, Gulbarga -585 106, India.

Correspondence:
Vijaykumar B. Malashetty
Department of Safety Assessment, Advinus Therapeutics Limited, Bangalore-560058, India

In vitro epiphyllous buds formation in *Enicostemma hyssopifolium* (willd.) ver. induction and cellular origin

Aravind Barad, Vijaykumar B. Malashetty and Seetharam Y. N.

Abstract
In vitro epiphyllous buds have developed on tender excised leaves of *Enicostemma hyssopifolium* (Willd.) Ver., cultured on Murashige and Skoog's (1962) medium supplemented with cytokinins (Benzyladenine and Kinetin). This phenomenon was shown to be due to cytokinins and occurred only when abaxial surface was in contact with the media. A two-day exposure to BA (2.0 mgl^{-1}) was sufficient for epiphyllous buds induction, although an exposure for more than four days yielded maximal shoot development from these buds. Culture of excised leaves on media supplemented with BA (2.0 mgl^{-1}) and subsequent subculture on hormone free medium was insufficient to induce epiphyllous buds formation indicating cytokinin dependence. Buds become microscopically visible after fourteen days. These epiphyllous buds originate from sub-epidermal cells on the adaxial side and consisted of functional meristems producing shoots formed by multicellular processes. The role of cytokinin and other potential factors in the induction and development of epiphyllous buds are discussed.

Keywords: epiphyllous buds, adaxial, asynchronous, micropropagation, benzyladenine, rooting

1. Introduction
Epiphylly is the occurrence of structures (leaves, shoots, inflorescence etc.,) upon a leaf or leaf homologue in any position (adaxially, abaxially, apically, marginally etc). Epiphyllous bud formation i.e., *de novo* morphogenesis on leaves of Angiosperms, occurs spontaneously under natural conditions in some monocotyledonous and many dicotyledonous species [1]. Epiphyllous buds can also be caused by parasitic insects on the leaves of certain species in which they do not occur naturally [2], and they can be induced by a range of *in vitro* culture treatments. The capacity to form epiphyllous buds *in vitro* is now widely exploited for the micro propagation of several species [3, 4]. Epihyllous buds formation has not been reported in Gentianaceae family to which *Enicostemma hyssopifolium* (Willd.) Ver, belongs. In *E. hyssopifolium*, the development of *in vitro* epiphyllous buds is peculiar in that it could be induced directly from the midrib of adaxial surface cultured under narrowly defined culture conditions. These conditions are likely to be the consequences of a variety of independent factors, which trigger cellular differentiation. The analysis of the process leading to this *de novo* bud formation was not the objective of the previous studies and hence there is no precise information available on the pattern of their development. Here we report on general conditions for induction and development of epiphyllous buds.

2. Materials and methods
A top cutting of 5-7 leaves emanating from perennial flowering plants collected during the month of August 2004 in a natural forest segment of botanic garden Gulbarga University, Gulbarga, Karnataka, India was used as an explant. The cuttings were washed in 1% (v/v) Labolene (Labolane India Pvt.Ltd., Mumbai) detergent for 5-7 minutes and then in running tap water for 15 minutes. Surface sterilization was done by immersion in 0.1% (w/v) mercury chloride for 3-5 minutes and followed by 4-5 washes in sterile distilled water. Leaf explants of 0.5-1.0 cm were dissected out washed once in sterile water and blotted over sterile filter paper discs before transfer to nutrient medium. The explants were implanted either vertically or horizontally with abaxial surface on the medium. The nutrient medium contains salts and vitamins of MS medium [5] supplemented with 3% (w/v) sucrose and varied concentration (1.0-2.5 mgl$^{-1)}$ of the BA or Kn and in combination with Indole-3 acetic acid (IAA) or ∝-naphthaleneacetic acid (NAA) (1.0-2.5 mgl$^{-1)}$ or indole-3 butyric acid (IBA).
The medium was adjusted to pH 5.7 before adding 0.8% (w/v) agar (Hi Media laboratories Pvt. Ltd., Mumbai), dissolved and dispend in culture tubes and then autoclaved at 121 ^{0}C and 1.1 Kgcm^{-2} pressure for 18 min.

All cultures were incubated in a culture room maintained at 25 ± 2 ^{0}C, RH 50-60% and 14 hr photoperiod, a photon flux density of 50-60 μEm^{-2}s^{-1}was provided by cool white fluorescent tubes (Philips India Ltd., Mumbai). The number of explants exhibiting epiphyllous buds formation was recorded under two categories stage I and stage II. Stage I, the adventitious buds are visible as dark green protuberances, in stage II the buds were clearly visible.

After 5 weeks the young shoots of nearly 1-2 cm were transferred to shoot multiplication and elongation medium containing different concentrations of BA (1.0-2.5 mgl^{-1}) to test the caulogenic response up to 5 subcultures each at 5 weeks intervals were tried. The epiphyllous buds and shoots were averaged and counted for 10 replicates and the experiment was repeated thrice.

For rooting, the shoots (~5 cm) were harvested and transferred individually to MS half and full strength agar or liquid medium with filter paper bridges containing 2% (w/v) sucrose and different concentrations of (0.5-2.5 mgl^{-1}) of NAA, IAA and IBA for rooting under 14 hours photoperiod.

The rooted plantlets obtained after 5 weeks were kept open for 5-6 days by loosening cotton plugs. After a day they were transferred to plastic cups with sterile inert supporting powder SOILRITE (Supplied by Karnataka explosive, India) and ½ strength liquid medium without sucrose. Each plantlet was covered with a glass beaker were maintained in the growth chamber at 90% relative humidity and 14 hours photoperiod. The well-developed plants were transplanted in pots containing a mixture of sterile soil, sand and farmyard manure (3:1:1). The humidity was gradually reduced to 60% over 20 days. The potted plants were well irrigated under green house conditions then transferred to the field successfully.

Statistical analysis was performed on the results of each experiment and the data were compared using analysis of variance (ANOVA) and Turkey test at 0.05% probability level.

To ascertain the nature of epiphyllous buds of differentiating structures were sectioned free handly or fixed in acetic acid alcohol (1:3) then dehydrated in ethanol- Xylol series embedded in paraffin wax and sectioned at 15 μ thickness and stained with haematoxylin and basic fuschin.

3. Results

The green leaves of 0.5-1.0 cm^{2} in length were reared on MS medium supplemented with different concentrations of BA and Kn (1.0-2.5 mgl^{-1}) and the responses are summarised in Table 1. The leaves cultured on MS medium with BA and Kn initiated *de novo* buds. The percentage of explant forming buds was dependent on the basal medium, cytokinin concentration and the type of cytokinin used. Within first 8 days of culture all the explants on media containing BA and Kn (1.0 - 2.5 mgl^{-1}) underwent slight swelling. By day 10, explants which had survived the initial culture period had swollen mid rib and edges. Epiphyllous buds are directly developed from the surface of the meristematic tissue (Fig. 1). Most of the buds are formed on different locations adaxially from main vein (Fig 1C) or from major secondary veins or from the cut ends of the explants (Fig 1D-H). But some arose abaxially from blade with no evident connection with the vascular bundles (Fig 1B). Phytohormones were the important factors affecting the rate of regeneration of *de novo* shoot buds from excised leaves (Table 1).

Shoot differentiation from leaf explants appears to be a function of cytokinin because auxin alone did not initiate shoot development. On MS medium with cytokinin alone epiphyllous buds were formed. These results presumably indicated a threshold level of endogenous auxin in the explant. Adventitious structures were first observed after 14 days with different frequencies. The frequency of epiphyllous buds formed increased with increase in the concentration of BA and Kn and at BA 2.0 mgl^{-1} the response was maximum and produce 100 per cent epiphyllous buds with 8.0 ± 0.25 shoot buds less than 1.0 cm length. Whereas Kn 2.0 mgl^{-1} produce 3.6 ± 0.45 buds with 60 per cent response and hence BA was preferred in all the further experiments.

3.1 General observations on epiphyllous buds formation

Single or multiple epiphyllous buds formed on MS medium from *in vivo* excised explants. Under optimal conditions at 2.0 mgl^{-1} BA, epiphyllous buds always formed on adaxial surface of the leaf. Formation of epiphyllous buds was asynchronous. Epiphyllous buds were observed together with proliferating and just arising buds on the same leaf (Fig 1C). Formation of the epiphyllous buds along the veins was often accompanied by alterations in leaf morphology. When epiphyllous buds arose on the main vein at the basal part of the leaf, the leaf frequently formed two pronounced lobes (data not shown). When several epiphyllous buds were distributed over the blade (more than two) but less pronounced lobes were usually formed. Epiphyllous buds formation was not always accompanied by lobe formation, but the formation of lobes was always accompanied epiphyllous bud formation.

3.2 Origin and development of epiphyllous buds

In order to estimate the time necessary for leaf cells to be induced and become determined towards epiphyllous buds formation, explants were transferred from BA containing medium to BA lacking medium and evaluated for epiphyllous bud formation after 35 days of culture (Table 1). The frequency of epiphyllous buds increased with increasing concentrations of BA and epiphyllous buds never formed on the leaves maintained on hormone free medium. However maximum number of epiphyllous buds was formed at BA (2.0 mgl^{-1}). Free hand section of epiphyllous buds cultured for 14 days indicated that only outer cell layers of leaf participate in the epiphyllous buds formation. However 5 days exposure to cytokinin itself is sufficient to produce maximal number of 22.2 ± 0.24 shoots with an average shoot length of 4.2 ± 0.32 cm formed per explant at 2.0 mgl^{-1} BA (Table 2) (Fig 2A). Epiphyllous buds develop into shoots after 35 days with several leaves established vascular connection with parental leaf (Fig 2B).

The earliest cytological observation indicating epiphyllous buds induction was found on 5th day of culture. By this stage few contiguous sub epidermal de-differentiating cells could be observed (Fig 3A). The nucleus of these cells was enlarged and contained a clearly visible nucleolus by 8th day (Fig 4A). Simultaneous division in epidermal and sub-epidermal cells produced small bumps covered with hairs, the cells of these bumps had characteristics of meristematic cells (Fig 3B) (Fig 4B). The functioning of this meristem (Fig 3D) then led to the production of foliar primordia ((Fig 3C). Thus shoots developed were rooted on ½ strength MS Medium supplemented with 1.0 mgl^{-1} NAA. It produced maximum number of 25.3 ± 0.36 roots per shoot with an average root length of 5.70 ± 0.09 cm after 35 days of culture and plantlet could be formed subsequently (Table 3) (Fig 2C).

Meristems never formed or developed from tissues situated in the abaxial part of the leaf, confirming the morphological

observation, which had indicated the exclusively adaxial position for epiphllyous buds formation. Epiphyllous buds at different steps of development to occur on the same sample. This indicates asynchrony either in the induction or in the development of the epiphyllous buds.

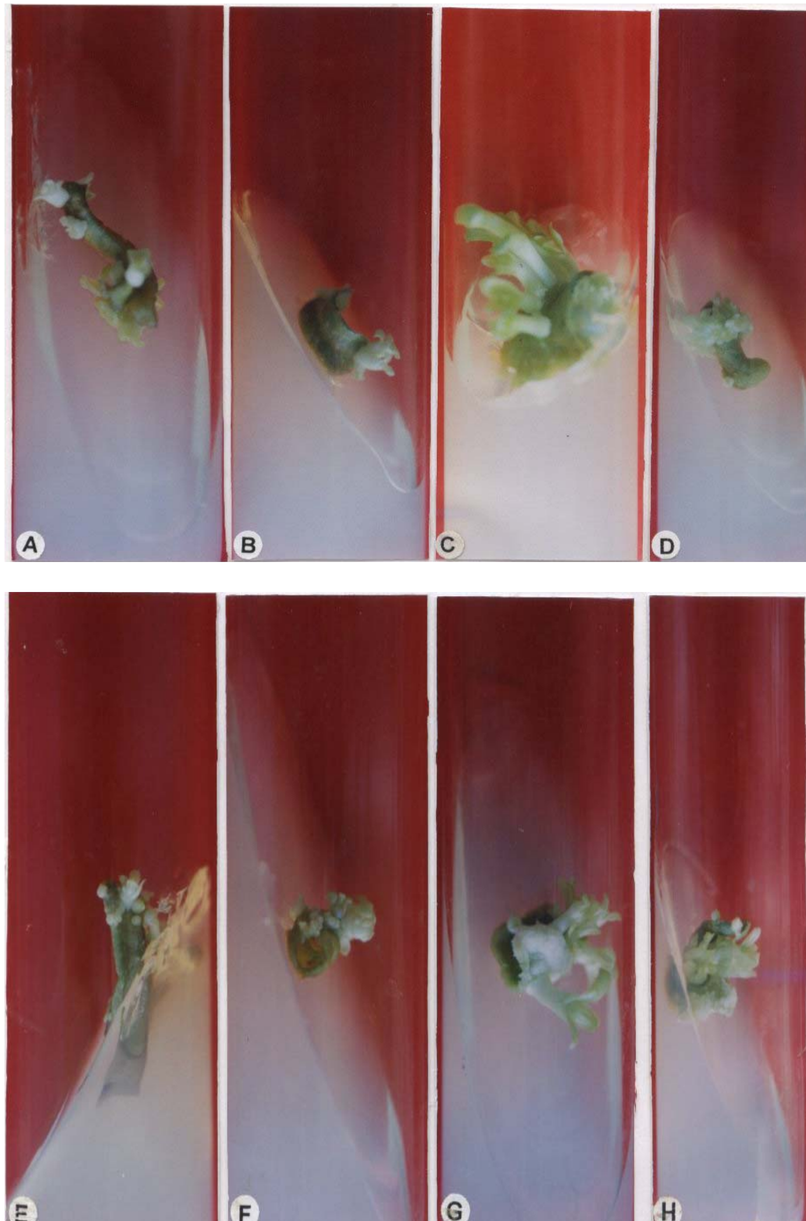

Fig 1: *In vitro* epiphyllous buds formation

A. Explant cultured for 14 days. Note epiphyllous bud formation on adaxial surface near main vein on MS + BA (1.5 mgl^{-1}).

B. Epiphyllous buds formation from abaxial surface after 15 days on MS + BA (1.0 mgl^{-1}).

C. Explant cultured for 35 days. Epiphyllous buds formation in clusters along the line on the main vein on MS + BA (2.0 mgl^{-1}) after 35 days.

D. formation from the cut ends of explant on MS + BA (1.0 mgl^{-1}).

E. Explants placed vertically on MS medium with Kn (1.0 mgl^{-1}). Note eb from adaxial surface along main vein and major secondary veins after 15 days.

F. Epiphllyous buds formation from the cut ends of adaxial as well as abaxial surface from cut ends on MS + Kn (1.5 mgl^{-1}).

G. Epiphyllous buds formation along the line on the main vein on MS + Kn (2.0 mgl^{1}).

H. Epiphyllous buds formation in cluster after 35 days on MS + Kn (2.0 mgl^{-1}).

Fig 2: Formation of epiphyllous shoots derived from epiphyllous buds of *E. hyssopifolium*

A. Maximal number of epiphyllous shoots after 35 days of culture.
B. Free hand section of cluster of epiphyllous shoots.
C. Rooting of epiphyllous shoots.

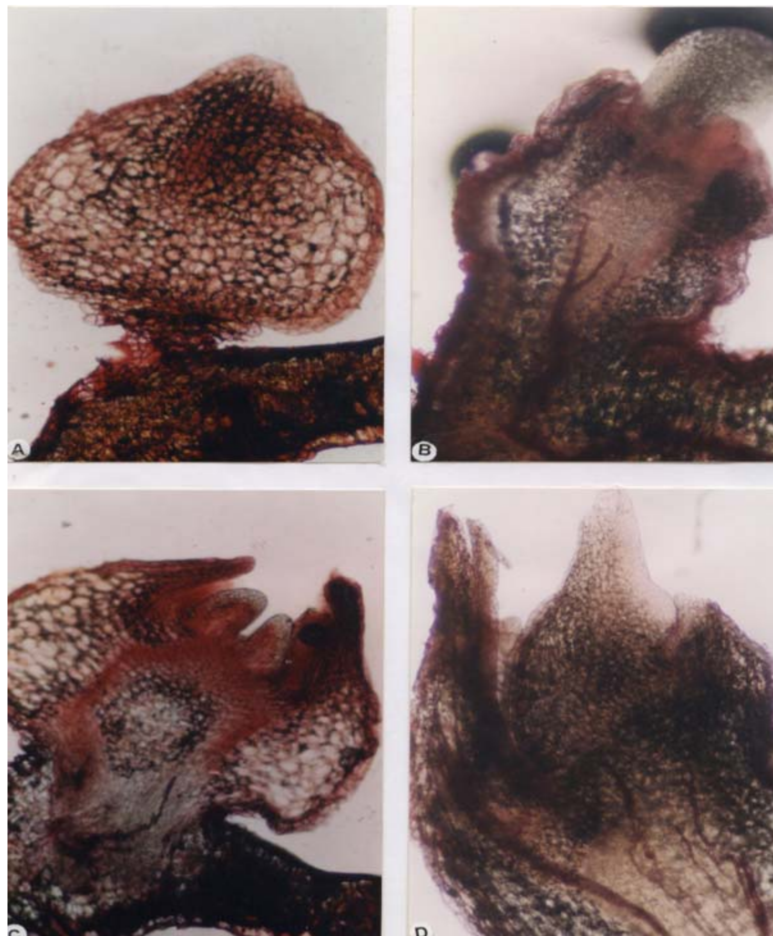

Fig 3: Free hand section of epiphyllous buds

A. Free hand section of a leaf cultured for 10 days, Note bumps on the adaxial surface on MS + BA (2.0 mgl^{-1}).
B. Free hand section of leaf cultured for 15 days produced meristem. Note the vascular connection with parental leaf.
C. Free hand section of leaf cultured for 20 days produced several leaves. Note the cotyledon development.
D. Formation of apical meristem with well-developed vascular connection with parent leaf.

Table 1: Effect of different concentrations of cytokinins on *in vitro* epiphyllous buds formation in *E. hyssopifolium*

Nutrient medium	Hormones (mgl^{-1})	Frequency of epiphyllous buds (%)	Days to buds	Location of buds	Average No. of epiphyllous buds
MS	BA (1.0)	40	19	Cut ends of Adaxial surface	1.9 (± 0.27)^{d}
MS	BA(1.5)	70	18	Adaxially and along the midrib	3.7 (± 0.49)^{bc}
MS	BA (2.0)	100	14	Adaxially and abaxially on secondary veins	8.0 (± 0.25)^{a}
MS	BA (2.5)	50	17	Abaxial lyon major secondary veins	4.9 (± 0.58)^{b}
MS	Kn (1.0)	30	25	Cut ends of adaxial surface	2.2 (± 0.63)^{d}
MS	Kn (1.5)	50	23	Secondary veins and along the midrib	2.9 (± 1.33)^{cd}
MS	Kn (2.0)	60	20	Adaxial surface from midrib	3.6 (± 0.45)^{bc}
MS	Kn (2.5)	40	22	Major secondary veins.	2.4 (± 0.89)^{d}

Data represent an average of 10 replicate cultures
Numbers of parentheses indicates ± SE
Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level.

Table 2: Effect of BA on epiphyllous shoots formation derived from epiphyllous buds of *E. hyssopifolium*

Nutrient medium	Hormones (mgl^{-1})	Average number of epiphyllous shoots	Average shoot length (cm)	Nature of shoots
MS	BA (1.0)	11.2 (± 0.38)^{c}	2.1 (± 0.43)^{a}	Greenish healthy with 1-2 internodes
MS	BA (1.5)	14.7 (± 0.30)^{b}	2.9 (± 0.27)^{a}	Greenish healthy with 2-3 internodes
MS	BA (2.0)	22.2 (± 0.24)^{a}	4.2 (± 0.32)^{a}	Greenish healthy with 2-4 internodes
MS	BA (2.5)	8.2 (± 0.33)^{d}	1.9 (± 0.50)^{a}	Greenish healthy with 1-2 internodes

Data represent an average of 10 replicate cultures (35 days old)
Numbers of parentheses indicates ± SE
Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level.

Table 3: Effect of auxins on rooting of excised shoots of *E. hyssopifolium* on ½ MS supplemented with 2% (w/v) sucrose

Sl. No.	Nutrient medium	Auxins (mgl^{-1})			%of shoots rooted	Average number of roots/ shoot	Days to rooting	Average root length (cm)
		IAA	NAA	IBA				
1.	½ MS	0.5	0	0	40	8.5(± 0.51)^{c}	18	2.80(± 0.45)^{bc}
		1.0	0	0	50	10.7(± 0.39)^{b}	16	3.01(± 0.31)^{b}
		1.5	0	0	60	13.8 (± 0.24)^{a}	17	3.45 (± 0.47)^{b}
		2.0	0	0	30	6.4 (± 0.34)^{d}	20	3.24 (± 0.37)^{b}
2.	½ MS	0	0.5	0.	50	13.3 (± 0.58)^{c}	19	5.38 (± 0.05)^{a}
		0	1.0	0	100	25.3 (± 0.36)^{a}	16	5.70 (± 0.09)^{a}
		0	1.5	0	80	16.1 (± 0.56)^{b}	16	5.55 (± 0.05)^{a}
		0	2.0	0	60	12.2 (± 1.36)^{c}	19	5.64 (± 0.07)^{a}
3.	½ MS	0	0	0.5	30	7.9 (± 0.37)^{b}	20	2.60 (± 0.40)^{d}
		0	0	1.0	40	9.3 (± 0.33)^{b}	18	2.76 (± 0.08)^{bc}
		0	0	1.5	50	10.9 (± 0.34)^{a}	20	2.70 (± 0.10)^{d}
		0	0	2.0	45	8.2 (± 0.50)^{bc}	23	2.70 (± 0.10)^{d}

Data represent an average of 10 replicate cultures (35 days old)
Numbers of parentheses indicates ± SE
Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level.

4. Discussion

Epiphyllous buds developing on the adaxial surface of *in vivo* excised leaves of *E. hyssopifolium*. This response only occurred when the explants had been subjected to cytokinin-supplemented medium. No epiphyllous buds were induced when the excised leaf segments were cultured on hormone-free medium. In all cases, the origin of epiphyllous buds was multicellular and was located in the sub epidermal cell layer. The presence of cytokinin for 5 days duration was sufficient for the proliferating cell clusters to become determined towards, the formation of shoot meristems which is visible after 14 days of culture. Maximal number of 8.0 ± 0.25 buds

with 100 per cent frequency was observed on MS basal medium supplemented with 2.0 mgl^{-1} BA.

Epiphyllous buds are known to occur on intact leaves in several species. They are generally of sub epidermal origin and emerge on the adaxial surface of the respective explants. Their induction is either endogenous and developmentally controlled (natural epiphylly) [6, 7] or is triggered by an elevation of cytokinin level. This cytokinin dependent induction can be provoked by exogenous treatment [8, 9]. Epiphyllous bud formation in our study, the appearance of small knobs along the main vein or prominent lateral veins was first observed after 14 days of culture. Epiphyllous bud formation always accompanied by hair growth originating from the epidermal layer. The phenomenon was also reported for natural epiphyllous buds in *Chisocheton tenuis* [6] and several tomato cultivars [7]. The reason for this co relation is still unknown.

The formation of adventitious meristems required the coordinated activity of several different cell types. First sub epidermal cells of no particular recognizable feature started to divide shortly afterwards, epidermal cells in the vicinity formed hairs at an unusual density. This activity was accompanied by occasional cell division in the epidermis. Later on the two dividing cell population become coordinated and together formed a functional meristem. This behavior requires doubtlessly a sophisticated system for cell-to-cell signaling, which may be related to the self-organizing and self-perpetuating capacity of shoot apical meristem [10].

Our experiments on the timing of epiphyllous bud induction showed that not only the induction step itself, but also further development of the epiphyllous shoots requires continuous external cytokinin supply. It is not unlikely that the epiphyllous bud clusters appears directly on, or in the close proximity of the leaf vein were induced by the exogenously added cytokinin transported in this vein. Further development of each individual epiphyllous bud within such a cluster requires continuous supply of cytokinin and is likely to be a competitive process. Maximal number of 22.2 ± 0.24 shoots with an average shoot length of 4.2 ± 0.32 cm were formed at 2.0 mgl^{-1} BA with 2–3 internodes. Single buds arising laterally on the leaf blade would be a subject to less competitive condition and therefore develop better.

Although exogenously added BA was required for epiphyllous bud induction, it must act through shoot apex similar to *Helianthus annuus* [11]. This observation could be explained by the necessity for the cytokinin to be supplied through vascular system. In addition to cytokinins there are several other factors required for the reaction. The KNAT1 gene alters hormone level that in turn causes veins to initiate ectopic shoots to form [12]. Auxin is thought to be a major regulatory factor in vasculature differentiation and variety of cytokinins promote xylem differentiation [13]. In addition, tobacco plants, transformed with the *Agrobacterium* iso pentyl transferases gene (Ipt), which leads to the synthesis of cytokinin, also produce ectopic shoots [12,14] from veins simply because KNAT1 expression is highest in that area [15,16] and revealed dramatic lobed phenotype [17]. Studies of the Knotted1 (Kn1) Homeobox gene of maize have shown that Kn1 is expressed in shoot portion of the plant and it is expressed very strongly in the meristem and down regulated before the initiation of leaf primordial [18]. It is therefore not unlikely in our experiments that epiphyllous buds formation is not only cytokinin dependent but additional factors required for meristem formation. It can be assumed that these factors are not normally present in the appropriate place and concentration but are mobilized by exogenously added cytokinin. Epiphyllous bud formation caused by exogenous cytokinin treatment (as observed in our experiments) and those reported for Knotted 1 over expression are very similar phenomenon [17, 19, 20].

5. Acknowledgements

One of the authors Aravind Barad thankful to Dr C.N Reddy, Gulbarga University Gulbarga, for providing facility to carry out this work.

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