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Research Article

Effect of Growth Regulators on Callus Induction and Organ Formation in *Cissus quadrangularis Linn.* A Valuable Medicinal Plant

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ABSTRACT

Cissus quadrangularis commonly known as 'bonesetter' is an ancient valuable medicinal plant documented in Ayurveda for the treatment of a number of diseases and disorders including healing of fractured bones. Its large scale propagation through conventional methods is beset with many problems and its over exploitation from wild necessitates a need to develop an efficient micropropagation protocol for its large scale multiplication. This valuable plant has not been studied much through *in vitro* technology and there are not many reports available on callus induction and hardly any report on its organogenic potential. In the present study, callusing was effected from the stem and leaf segments taken from field grown mature plant on MS medium supplemented with different concentrations of Naphthelene Acetic Acid (NAA) and Kinetin (Kn) with the highest callus growth occurring on 5.37 µM NAA and 2.32 µM Kn. Further addition of 15% CM and 3% glucose (instead of 2% sucrose) to the medium considerably enhanced the callus growth and delayed its browning on subsequent subculturing. The calli formed were yellowish white, highly friable and heterogenous in nature. The histogenetic differentiation in the form of tracheids occurred in all the calli followed by root differentiation on higher concentrations of NAA(10.74-21.48 µM)and Kn (4.65 µM) after 4-5 weeks. A low frequency shoot differentiation from leaf callus was observed on NAA (14.7µM) and Kn (2.32µM) after 8 weeks. Direct rooting from leaf and stem segments was observed on MS medium supplemented with different concentrations of auxins like Indole 3 Butyric Acid (IBA) or NAA either alone or in combination with kinetin.

Keywords: *Cissus quadrangularis,* callus, differentiation, micropropagation, Murashige and Skoog

INTRODUCTION

Cissus quadrangularis Linn. belonging to family Vitaceae is an ethno-pharmacologically important medicinal plant commonly known as "bone setter" and is referred to as ''Asthisamdhani' in Sanskrit and 'Hadjod' in Hindi because of its specific ability to join fractured bones[1]. The plant has been prescribed in the ancient system of Ayurvda as a general tonic especially for the patients with fractured bones. It has numerous medicinal uses and is effective for the treatment of a plethora of diseases ranging from gouts, diarrhea, skin disorders, irregular menstruation, piles, tumors, scurvy, hemorrhoids, rheumatoid arthritis and bone healing[2-5]. It is also used for obesity, diabetes, a cluster of heart disease risk factors called "metabolic syndrome", high cholesterol and also used in body building supplements as an alternative to anabolic steroids[6]. The entire plant is of medicinal value and is known to contain many phytochemical constituents having potentially diverse therapeutic effects. Phytochemical analysis reveals an array of bioactive compounds such as steroids, carotene, flavanoids, triterpenoids, ascorbic acid and calcium[7,8] .

Cissus is reported to contain a number of steroids including β-sitosterol, daucosterol, β-amyrin,δ-amyrone, tarexerol and freidalin[9-13]. Besides, a number of stilbene derivatives like Quadrangularis A, B and C and resveratol, piceatannol, pallidol and parthenocissin are also reported to be present in the stem[14,15]. *C. quadrangularis* is an edible plant which grows natively in the hotter and dried regions of India such as Deccan Peninsula and is also found on lower eastern slope of Western Ghats. It is also wide spread in the drier areas of the world such as Pakistan, Bangladesh, Sri Lanka, Malaya, Thailand, Java and west Africa[16]. It is a succulent perennial climber having slender, fleshy, fibrous, quadrangular and jointed stem with constrictions at its nodes. Leaves are simple, alternate, fleshy 3-lobed ovate with serrated margins and numerous tendrils grow out of the plant's nodes. The flowers are small, bisexual, tetramerous, greenish white occurring in short umbellate cymes opposite to leaves and come at the end of the summer. *C. quadrangularis* is conventionally propagated vegetatively through stem cuttings and that too mainly in the rainy season. But it is a slow, cumbersome and inefficient procedure due to less

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Figure 1 A. Direct root induction from stem segments on MS + IBA(4.98µM) after 20 days of culturing **B.** A leaf segment regenerating roots on MS + NAA (10.74 µM) + Kn (2.325 µM) after 15 days **C.** A shoot regenerating directly from the stem segment on MS + 2,4-D (19.48µM) **D.** Callus initiation at the cut ends of a stem segment on MS+NAA (5.37µM) + Kn (2.32µM) or NK medium **E.** Formation of yellowish white and friable callus on NK medium after 4 weeks **F.** Whole stem segment transformed into mass of callus on NK medium + 15% CM + 3% glucose after 3 weeks **G.** Root differentiation from stem callus on MS + NAA (21.48 µM) + Kn (4.65 µM) after 6 weeks

number of propagules obtained. Propagation through seeds is unreliable as flowers are invariably sterile and do not set seeds. In view of its growing demand and the difficulties associated with its conventional propagation, it is necessary to look for an alternate method having high multiplication rate. In this context, *in vitro* technology holds significance promise for its conservation and to meet the growing and sustainable supply of high quality planting material for pharmaceutical industry. To the best of our knowledge this valuable plant has not been studied much through tissue culture technology and except 2-3 fragmented reports on forced axillary branching, not much reports are available on its micropropagation [17,18]. In our previous report, we reported the first successful protocol for the micropropagation of *C.quadrangularis* from the nodal segments and shoot apices taken from field grown mature plant [19]. The present investigation deals with the induction and growth of callus from the stem and leaf segments of mature native plant and thereafter to explore the potentialities of the calli for organogenesis.

MATERIAL AND METHODS

Initiation of Aseptic cultures

Stem and leaf segments were collected from a field grown healthy and mature plant of *Cissus quadrangularis*. These were placed in glass bottles, covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles from the surface. The explants were then washed with liquid detergent (1% v/v) for another 10-15 minutes followed by their repeated washings with water.

Table 1: Effect of different PGRs on callus induction from stem segments

S. No.	Plant Growth Regulators	% of Explants formed callus	Time taken to initiate callus (Days)	Degree of callusing
1	MS+NAA(5.37µM)	40	10	+
2	MS+NAA(10.74µM)	40	10	+
3	MS+NAA (5.37µM)+Kn(2.325µM)	90	8-10	++++
4	MS+NAA (5.37µM)+Kn(4.65µM)	80	10-12	+++
5	MS+NAA (10.74µM)+Kn(2.325µM)	75	10-12	+++
6	MS+NAA (10.74µM)+Kn(4.65µM)	75	15	+++
7	MS+NAA(5.37µM)+Kn(2.325µM)+15% CM	100	8-10	+++++
8	MS+NAA(5.37µM)+Kn(2.325µM)+15% CM+3% sucrose	100	8-10	+++++
9	MS+BAP (8.88µM)	30	25	+++

"+++++" very good growth, ,"++++" good growth,"+++" average growth , "+" very less growth

The explants were thereafter treated with bavistin (0.1 % w/v) for 10-14 minutes to remove the fungal contamination and then washed thoroughly to remove the fungicide. Final disinfection was done with an aqueous solution of 0.1% mercuric chloride ($HgCl_2$) for 2 minutes for leaves and 3-4 minutes for stem segments in a laminar air flow cabinet. The explants were then thoroughly rinsed (4- 5 washings) with sterilized distilled water to remove the traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions. Thereafter, they were planted on Murashige and Skoog's medium (1962) [20] referred to as MS medium augmented with different growth regulators, 2% sucrose or 3% glucose and 1% agar. Twenty replicates were set for each treatment and each experiment was repeated twice.

Callus Induction and Differentiation

The sterilized leaves and stem segments were inoculated onto MS medium supplemented with different auxins like NAA (2.68-21.48 µM), IBA(4.90-19.60 µM), Indole-3-Acetic Acid or IAA(5.71-22. 84 µM), 2,4-Dichlorophenoxy Acetic Acid or 2,4-D (2.26-18.12 µM) either alone or in conjunction with cytokinins like Kn (2.32- 18.60 µM) and BAP or Benzyl Amino Purine (2.22 to 17.76 µM) for callus induction and direct organ regeneration. The pH of the medium was adjusted to 5.8 ± 0.1 and the medium was solidified with 1% (w/v) agar and autoclaved at 121ºC and 1.05 kg/cm-^{2} for 15 minutes. Callus was shifted to various combinations of cytokinins and auxins for inducing differentiation of shoots and roots and best combinations for optimum response were selected.

Culture incubation

All the inoculated cultures were incubated in growth room at a temperature of 25 ± 2ºC with a photoperiod of 16 hours per day with an illumination of 50 µmol/m-2/s-1 at the level of cultures..

RESULTS

Direct organ induction

Direct rooting from stem segments was observed on MS medium supplemented with different concentrations of IBA, 2, 4-D and NAA either alone or in conjunction with Kn. Best rooting response occurred on 4.90µM of IBA where 3-5 roots were formed within 20 days of inoculation (Fig. 1A). The roots were long, white and without root hair. Direct rooting was also observed on different concentrations of 2,4-D (4.53—18.12 µM) and higher concentration of NAA (10.74

Table 2: Effect of different PGRs on callus induction from leaf explants

S. No.	Plant Growth Regulators	% of Explants formed callus	Time taken to initiate callus (Days)	Degree of callusing
1	MS+NAA(5.37µM)	20	15	+
2	MS+NAA(10.74µM)	20	15	+
3	MS+NAA(5.37µM)+Kn(2.325µM)	100	15	+++++
4	MS+NAA (5.37µM)+Kn(4.65µM)	80	15	++++
5	MS+NAA (10.74µM)+Kn(2.325µM)	80	15	++++
6	MS+NAA (10.74µM)+Kn(4.65µM)	80	18	++++
7	MS+2,4-D (4.53µM)+Kn(4.65µM)	20	20-25	+
8	MS+2,4-D (9.06µM)+Kn(9.30µM)	20	20-25	+
9	MS+BAP (4.44µM)	20	25	+
10	MS+BAP (8.88µM)	30	25	+

"+++++" very good growth, ,"++++" good growth, "+" very less growth

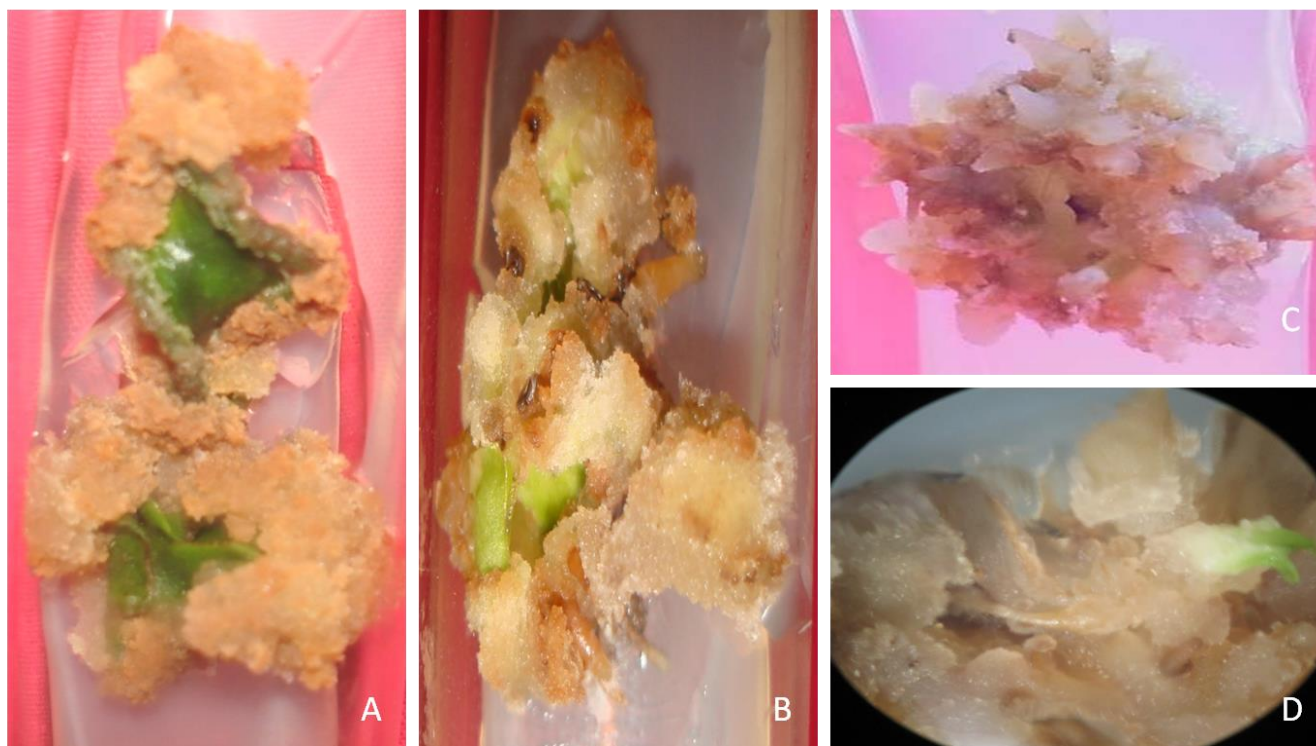

Figure 2. A.&B. Leaf explants callusing on NK medium after 2 and 4 weeks of culturing **C.** Numerous roots differentiating from leaf callus on MS + NAA (21.48 µM) + Kn (2.325 µM) after 6 weeks **D.** A shoot bud (arrow) differentiated from leaf callus on MS + NAA (14.7µM) + Kn (2.325µM) after 8 weeks of culturing

µM) along with Kn (2.32-4.65 µM) where 1-2 roots were formed per explant. Leaf explants regenerated roots on MS medium containing NAA (10.74 µM) and Kn (2.32 µM) within 15 day of culturing. Rooting occurred either at the cut ends or along the surface and roots formed were white, thick , unbranched and bore no root hairs. (Fig.1B). With higher concentrations of NAA (21.48 µM) and Kn (9.30 µM) fortification, rooting was pronounced but callus induction occurred simultaneously with rooting. However, no rooting from leaf explants was observed on media containing IBA, 2, 4-D or IAA either alone or in combination with Kn.

Direct adventitious shoot regeneration from the stem segment occurred on MS medium supplemented with 2, 4-D (18.12µM) after 20 days of culturing in about 1% of cultures Figure 1C depicts a shoot regenerating from the stem segment after 4 weeks.

Callus Induction and Growth

Different Auxins (NAA, IBA, and 2, 4-D) and Cytokinins (Kn and BAP) alone or in combination were supplemented in various concentrations for establishing callus from the stem and leaf explants. Although addition of NAA (5.37 µM - 21.48 µM) to the medium induced callus successfully, but the addition of Kn enhanced the callus formation many folds. Good callusing from the stem segment occurred on NAA (5.37µM) and Kn (2.32 µM) hereby designated as NK medium where 90 % of the explants callused either at the cut ends or along the entire surface after 8-10 days of culturing (Fig. 1D) and within 4-5 weeks, the entire explant turned into a mass of yellowish white and friable callus (Fig. 1E). Further, the addition of CM (15%) to the NK medium demonstrated prolific callusing from the stem segments. On this medium callusing initiated earlier and the explant turned into mass of callus within 3-4 weeks. The callus, however, did not show sustained growth on NK or coconut supplemented media and turned brown on subsequent sub-culturing. In order to delay or stop browning, callusing was induced on the above medium with 3% glucose instead of 2% sucrose. Good quantity of callus was formed on NAA (5.37µM)+Kn (2.32µM)+15% CM+3% glucose (Fig.1F). The callus was whitish in colour and showed sustained growth and a marked decrease in browning was observed with subsequent sub culturing. Callus induction from stem segment was also observed on BAP (4.44 µM-17.76 µM) supplemented medium after 28 days of inoculation but the rate of callus formation was slow and the callus formed was compact, hard and greenish brown in color. Effect of different growth hormones on callus induction from stem segments is shown in table 1. Like stem segments, best callusing from leaves also occurred on NK medium where callusing started from the cut ends after 15 days of culturing (Fig.2A) . The callus proliferated further (Fig.2B) and after 5 weeks, the whole leaf segment turned into a mass of yellowish white, soft and friable callus. The callus proliferated further on subculturing showing sustained growth. MS medium supplementation with 2,4-D (4.53 µM-9.06 µM) or BAP (4.44 µM-8.88 µM) alone and in

combination with Kn (4.65 µM-9.30 µM) also resulted in callus formation but callus growth was very slow and poor. Different concentrations and combinations of growth regulators used and their effect on callus induction from leaf explants is depicted in table 2.

Study of callus and Histogenetic Differentiation

The stem and leaf calli raised on NK medium were highly friable, breaking up into single cells or group of cells when placed in water. The calli ware heterozygous comprising of cells of different shapes and sizes. Cells were spherical, ovoid and elongated having prominent nuclei and numerous starch grains. Histogenetic differentiation in the form of tracheids occurred in both the calli showing reticulate thickenings on their walls. The tracheids were present either singly or in groups forming nodules.

Organogenetic differentiation from callus

Root differentiation from the stem callus occurred on NAA (10.74-21.48 µM)and Kn (4.65 µM) augmented medium after 6-7 weeks of culturing. Initially 3-4 roots were formed from the callus (Fig.1G) but with further proliferation more and more roots differentiated. Rhizogenesis from the leaf callus was observed on MS medium containing different concentrations of NAA (5.37µM-21.48 µM) along with Kn (2.32 µM) after 5 weeks of culturing with the highest response on NAA (21.48 µM) and Kn (2.32 µM). The roots were small, thick and white without hair (2C). No differentiation of roots was seen on IBA or IAA supplemented medium. In the present investigation, a low frequency shoot differentiation from leaf callus was also observed in 2% of the cultures on NAA (14.7µM) and Kn (2.32µM) after 8 weeks of culturing but they failed to develop into proper shoots. Figure 2D depicts the differentiation of a shoot initial from the leaf callus. However no shoot differentiation could be effected from the stem callus on any of the media combination tried.

DISCUSSION

Majority of the plant tissues growing *in vitro* require exogenous hormones in the nutrient medium for dedifferentiation. Our experiments clearly demonstrated that the nature and concentrations of auxins and cytokinins and other growth substances have marked influence on callusing of the explants. The synergistic action of NAA and Kn (NK medium) was demonstrated for the initiation and sustained growth of callus from the stem and leaf segments. However both the organs exhibited differential sensitivity to exogeneosly supplied chemical stimuli. While leaf segments responded best on NK medium, stem segments showed best callusing on supplementation of NK medium with CM and glucose instead of sucrose in the medium. The results can be explained on the basis that different plants and even different organs of the same plant are characterized not only by their unique intrinsic biochemical make up but also by the sensitivity to the exogenously supplied chemical stimuli. The reaction of an isolated tissue to auxin depends upon its endogenous auxin level at the time of excision and its genetic capacity for its synthesis. Earlier Sharma et al. [21] had reported callus formation from nodal segments of *Cissu quadrangularis on* MS medium supplemented with 3% sucrose, 2.5mg/l NAA and 0.5mg/l BAP and further evaluated the role of auxins in the enhanced synthesis of sterols. Induction of greenish yellow friable embryogenic callus from leaf segments of *C.quadrangularis* has been reported on MS salt, Gamborg B5 vitamins, 2,4-D and BAP supplemented medium[22]. A slow growing callus from the stem segments of *C.quadrangularis* on different concentrations of NAA and 2,4-D formed after 90-120days has been reported by Teware et al[23] .Although 2-3 reports are available on the callus induction, but there is hardly any report available on organ regeneration either directly from the explant or indirectly through callus in *C.quadrangularis.* The current work reports for the first time direct root regeneration from stem and leaf explants when cultured on MS medium supplemented with either of the two auxins IBA or NAA and Kn. Organogenesis from stem and root calli were observed in the form of roots on higher concentrations of NAA(10.74-21.48) and Kn(2.32-4.65). Initially a few roots were formed but with further proliferation of the callus more and more roots were organized. Similarly Kaur [24] reported NAA at a high concentration of 29.4 µM with 4.65 µM of Kn to be the most effective growth regulator for inducing rhizogenesis from leaf and stem calli of *Tylophora indica*. A very meager shoot bud differentiation from the leaf callus occurred on NAA (14.7µM) and Kn (2.32µM) augmented medium in 2% of cultures but they failed to develop into proper shoots on any of the media combination tried.

CONCLUSION

A successful protocol for the induction and proliferation of callus from intermodal and leaf segments of *Cissus quadrangularis* has been established which exhibited root differentiation in 40% cultures. A low frequency shoot bud differentiation (caulogenesis) occurred in 2% of callus cultures but they failed to develop into proper shoots on any of the media tried. It is firmly opined that the cells are undoubtedly totipotent but some vital hormonal and/or nutritional factor triggering shoot elongation could not be discovered by us during the stipulated period of this study.

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CONFLICT OF INTEREST

It is hereby declared that there is no conflict of interest.

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