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Somatic Embryogenesis and *In vitro* Plant Regeneration in *Vigna trilobata* (L.) Verd. - A Potential Pasture Legume

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Abstract

This long term study demonstrates for the first time that it is possible to propagate embryogenic *Vigna trilobata* and to subsequently initiate the differentiation of embryos into complete plantlets. Initiation of callus was possible on 2,4-D. Somatic embryos differentiated on modified MS basal nutrient medium with 1.0 mg/l of 2,4-D and 0.5 mg/l of Kn. Sustained cell division resulted in globular and heart shape stages of somatic embryos. Transfer of embryos on to a fresh modified MS basal medium with 0.5 mg/l of Kn and 0.5 mg/l of GA_3 helped them to attain maturation and germination. However, the propagation of cells, as well as the differentiation of embryos, were inhibited by a continuous application of these growth regulators. For this reason, a long period on medium lacking these growth regulators was necessary before the differentiation of embryos occurred again. The consequences for improving the propagation of embryogenic cultures in *Vigna* species are discussed.

Introduction

The genus *Vigna* has more than 200 species of which several have considerable economic importance in many developing countries. They can be grown successfully in extreme environments (e.g., high temperatures, low rain fall, and poor soils) with a few economic inputs. *Vigna trilobata* (L.) Verd. is a wild species belonging to Fabaceae. Sumanasinghe and Tomooka 1997 considered that *V. trilobata* is a wild ancestor of moth bean (*V. aconitifolia*). It is commonly called as African gram (English); Jangli moth (India); mukni (Pakistan). It is primarily a regenerating annual herb, with glabrous pubescent stems, prostrate and trailing.

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V. trilobata can be distinguished from the morphologically similar *V. aconitifolia* by virtue of large oval stipules, the latter having small, linear-lanceolate stipules. This plant is native to semi-arid regions of Asia. *V. trilobata* is largely found on well-drained, alkaline, dark, cracking clay soils, but also on sandy and loamy soils of similar reaction (pH 6.5 - 9). Mainly occurs in grassland, on road verges, irrigated land, drain edges and banks of irrigation channels. It has high potential to be used as forage (Hacker and William 1996). *Vigna trilobata* is sown in India as a short term pasture and green manure crop (Jain and Mehra 1980). *V. trilobata* had a higher content of crude protein than the commonly consumed Indian pulses (Arinathan et al. 2003). Some tribes in India collect wild *V. trilobata* and used as human food, the pods being eaten as a vegetable and seeds are cooked and eaten, have a good nutritional value (Siddhuraju and Vijayakumari 1992).

Considerable research work has been done on cowpeas (*V. unguiculata*) and mung bean (*V. radiata*) in the United States and Europe during the last century. However, limited attention is paid to other *Vigna* species. This situation is now changing. Shekhawat and Galston (1983) reported culture and regeneration of *V. aconitifolia* plants from isolated protoplasts. Kaur-Sawhney et al. (1985) reported that aseptically cultured mesophyll protoplasts of *V. aconitifolia* divide rapidly and regenerate into complete plants. Plant regeneration from cell suspension cultures of *V. aconitifolia* was reported by Kumar and Gamborg (1988). Ignacimuthu et al. (1997) and Das et al. (1998) found cotyledonary nodes of *Vigna* have been most responsive for the induction of multiple shoots via organogenesis. Induction of Somatic embrogenesis via suspension culture in *Vigna* species was reported by Prem Anand et al. (2000). *Agrobacterium* mediated transformation of cotyledonary node explant has been reported in *Vigna* species. by Ignacimuthu (2000). Shoot regeneration from various *Vigna* species was studied by Renato et al. (2001). Chandra and Pental (2003) reviewed regeneration and genetic transformation of grain legumes including *Vigna* species. Developmental pattern formation of somatic embryo induced in cell suspension cultures of *Vigna unguiculata* was reported by Ramakrishnan et al. (2005). Saini and Jaiswal (2005) reported transformation of a recalcitrant grain legume, *Vigna mungo* using *Agrobacterium tumefaciens* in shoot apical meristem cultures.

In recent years genetic engineering has been effectively used to develop desirable breeding lines of many important crop plants (Fisk and Dandekar 1993, James 2004, Wambugu 1999). A reproducible and reliable transformation system enables us to insert genes which are not available in the existing genotypes. Thus, it may be possible that, genetic transformation combined with traditional breeding may prove helpful in improving both the quality and yield of *Vigna* beans. However, the *in vitro* regeneration protocols and tissue culture work on *Vigna trilobata* have not been reported by any worker. Thus, in the present

investigation attempts were made to establish *in vitro* plant regeneration system for *Vigna trilobata*.

Materials and Methods

Plants of *Vigna trilobata* (L.) Verd. were collected from Udaipur and Sikar, India. The experiment was performed using different explants of the *V. trilobata*. Mother plants were also grown in a greenhouse at 28ºC under a 14/10 hrs (light/dark) photoperiod with light intensity of 50 μmol/m^{-2}/s. Various parts of plants *viz.* juvenile leaves, stem cuttings, roots and immature seeds were used as source of explant.

Juvenile leaves from mature plants were initially cleaned with 0.5 % (w/v) solution of detergent, sodium dodecyl sulfate (SDS) for 15 min. These were thoroughly washed with autoclaved sterile water. These explants were then treated with 0.2 % (w/v) solution of systemic fungicide, Bavistin. Explants were surface sterilized with aqueous solution of 0.2 % (w/v) $HgCl_2$. These were then washed with autoclaved water for several times and used as explant.

Each treatment included five explants, and was not repeated. Instead of replications, the experimental scheme was extended to different combinations of growth regulators to allow a comprehensive assessment of responses. Each explant was cultivated in a 50 ml test tube containing 10 ml medium. The surface sterilized juvenile and young leaves were dissected lengthwise in order to initiate the callus from the cut ends. Dissected leaves were aseptically placed on basal MS and modified MS (Shekhawat et al. 1993) medium gelled with 0.8% agar, 3% sucrose and various concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) of 2,4-D or NAA or 2,4-D/Kn. The test tubes were sealed with cotton plugs and incubated in a growth chamber at 26 ± 2ºC under a 14/10 hrs (light/dark) photoperiod with light supplied by white fluorescent lighting at an intensity of 46 μmol/m^{-2}/s. The callus initiation was also tried on experimental media, namely full strength Whites's and AMS media. Each of the medium was supplemented with 2.0 mg/l of 2,4-D. The callus was subcultured after 20 - 25 days on MMS medium containing 2,4-D ranging from 0.1 to 2.0 mg/l. Experiments were designed to optimize the culture conditions for the induction of organogenesis or embryogenesis. The primary culture was followed by five subcultures of four weeks each on the same medium. Multiplied callus was subcultured on to MMS supplemented with combination of 1.0 mg/l of 2,4-D with different concentrations (0.25 - 1.0 mg/l) of BAP, Kn and 2-iP for induction of somatic embryogenesis.

For growth characterization, the fresh mass of the cultures was measured after the primary culture and after each of the first five subcultures on the medium containing growth regulator. In addition, the initial value of fresh mass

was determined using leaf segments from a sample of 30 explants. Experiments were designed to optimize *in vitro* conditions for the purpose of maturation of embryos. Both the liquid and agar gelled media were evaluated. The effect of the media composition was tested in terms of percentage of callus converting into mature embryos and number and length of plantlets regenerating from mature embryos. Effect of low concentrations of each of BAP and Kn was tested. And also various combinations of GA_3 with BAP and Kn were evaluated.

The histological examination was performed as described by Haensch (2004). The toluidine blue stains the cytoplasm and unlignified cell walls red and the DNA-containing structures and lignified cell walls blue. Embryogenic callus was transferred on to agar gelled MMS supplemented with additives, 3% sucrose, and 0.5 mg/l each of Kn and GA_3 acid incubated under the cool white fluorescent light at 26 ± 2^0C in the culture room. The cultures were maintained in 150 ml flasks.

The somatic seedlings of *V. trilobata* thus produced were then transferred to glass bottles containing soilrite moistened with 1/4th strength of MS macro salts. These bottles were kept in greenhouse at 27 ± 2°C and a relative humidity of 70 - 80%. These seedlings after hardening were transferred to black polybags containing sandy soil.

Results and Discussion

Callus induction and maintenance have been difficult task to achieve in leguminous plants, *Vigna* are no exception. Of the different explants evaluated for the establishment of cell cultures of *V. trilobata*, the small juvenile immature leaves were found to be the most suitable. The explants were placed on MS and modified MS containing 2 mg/l of 2,4-D. Callus proliferated from the margins of inoculated leaves on modified MS was creamy yellow and fragile (Fig. 1). The primary callus initiated the callus on MS and modified MS + 0.5 - 1.5 mg/l 2,4-D, it turned watery and unorganized. Modified MS supplemented with 2.0 mg/l 2,4-D was found best for callus initiation (Table 1).

Experiments were conducted to induce organogenesis or embryogenesis in the cultures. Most of the growth regulator combinations have given rise to the formation of globular embryos. The histological examinations revealed that the globular stage somatic embryos are new individuals, which consist of small cells with a dense cytoplasm and develop to bipolar structures showing clearly well developed shoot and root meristems. (The callus cultures exhibited poor growth on lower concentrations (0.1 - 0.5 mg/l) of 2,4-D. On transferring the initiated callus on MMS + 1.0 mg/l 2,4 D, it became granular pale yellow in color and was highly regenerative (Fig. 2). Use of auxin in combination with cytokinin proved to be favorable for somatic embryogenesis. Callus transferred on to 1.0 mg/l of

Table 1. Effect of different concentrations of auxin and cytokinin on callus initiation on modified MS and additives in *Vigna trilobata*.

PGR treatment (mg/l)	Percentage of response	Days taken for callus intiation
2,4-D		
0.5	20	18
1.0	35	18
1.5	70	16
2.0	75	15
2.5	65	15
NAA		
1.0	-	-
2.0	10	25
Kn		
0.5	10	25
1.0	10	25
2.0	-	-
2,4-D +Kn		
1.5 + 0.5	40	20
1.5 + 1.0	30	25
2.0 + 0.5	45	18
2.0 + 1.0	40	20

Table 2. Effect of different concentrations and combinations of auxin and cytokinin on callus growth and induction of somatic embryos on modified MS additives in *V. trilobata*.

PGR treatment (mg/l)	Average No. of SE per culture vessel ± SD
2,4-D + Kn	
1.0 + 0.25	25 ± 0.75
1.0 + 0.50	55 ± 0.88
1.0 + 0.75	35 ± 1.27
1.0 + 1.00	10 ± 1.02
2,4-D + BAP	
1.0 + 0.25	15 ±1.35
1.0 + 0.50	20 ± 1.47
1.0 + 1.00	-
2,4-D + iP	
1.0 + 0.25	10 ± 1.65
1.0 + 0.50	-
1.0 + 1.00	-

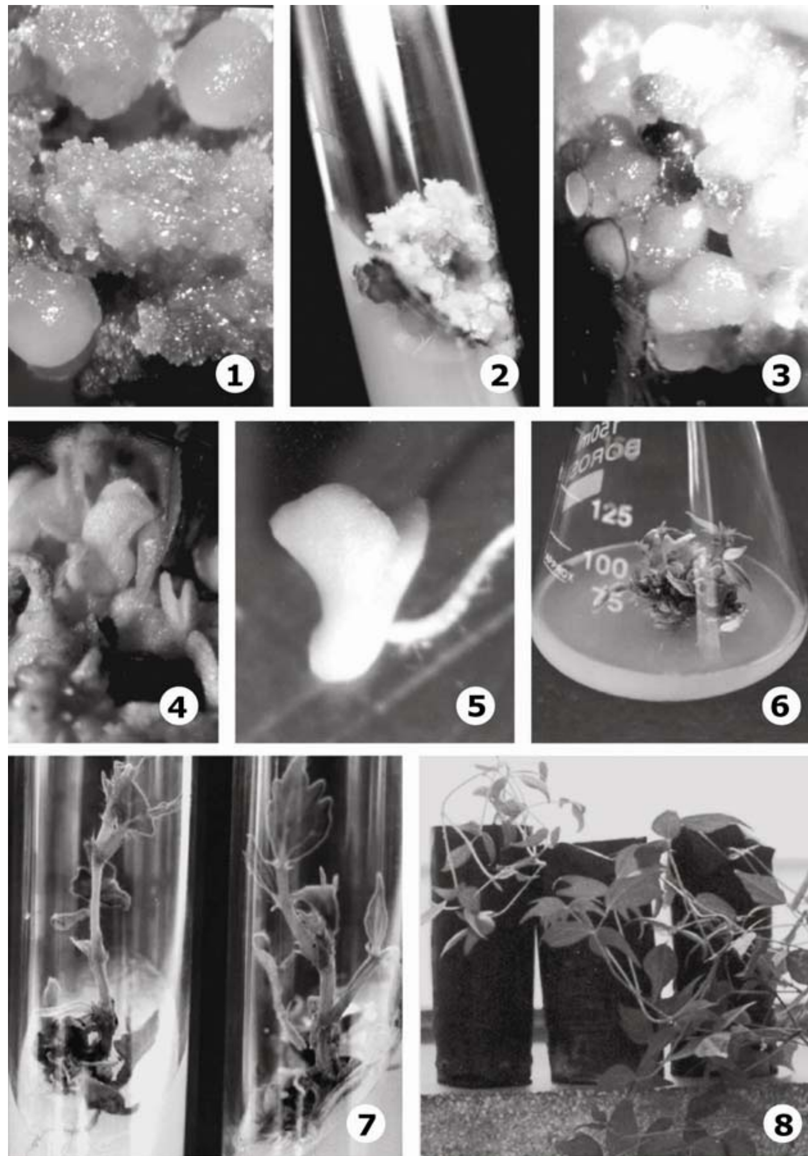
2,4-D and 0.5 mg/l of Kn differentiated in small, green globular embryos (Fig. 3). Higher concentration of Kn resulted in loss of organogenetic potential (Table 2.)

Prolonged maintenance of the callus on the same medium resulted in the loss of potential and the growth of the green competent cells was surpassed by the non-embryogenic callus. On subculture to growth regulator free MS medium the embryogenic potential was lost. Thus soon after the appearance of green globular embryos the callus was transferred on growth regulator containing medium. Growth and multiplication of the initiated embryos occurred on agar-gelled MMS supplemented with 0.5 mg/l Kn and 0.5 mg/l GA_3 (Figs. 4, 5; Table 3). All the embryos differentiated and attained maturity. Use of different growth regulators in varied concentrations affected the number as well as length of plantlets regenerated. Ten - 12 plantlets of 4 - 5 cm in length differentiated from each vessel on MMS supplemented with 0.5 mg/l Kn and 0.5 mg/l GA_3 (Figs. 6 and 7).

Table 3. Effect of different concentrations and combinations of plant growth regulators on plant regeneration on modified MS in *Vigna trilobata*.

PGR treatment (mg/l)	Shoot number/culture vessel ± SD	Shoot length (cm) ± SD
HF	1.4 ± 0.51	1.43 ± 0.38
BAP		
0.05	1.5 ± 0.52	2.27 ± 0.40
0.25	1.4 ± 0.51	3.38 ± 0.51
0.50	1.6 ± 0.51	2.34 ± 0.45
Kn		
0.05	1.6 ± 0.51	3.06 ± 0.67
0.25	2.4 ± 0.51	4.02 ± 0.60
0.50	2.6 ± 0.51	5.04 ± 0.62
1.00	1.3 ± 0.48	5.41 ± 0.28
GA_3		
1.0	-	
Kn+ GA_3		
0.5+0.5	11 ± 0.94	4.53 ± 0.36
BAP + Kn		
0.5 + 0.5	5.5 ± 0.52	3.44 ± 0.25
0.5 + 1.0	3.4 ± 0.51	4.44 ± 0.34
1.0 + 1.0	1.7 ± 0.48	2.55 ± 0.32

The somatic seedling regenerated through cell culture regeneration was transferred to bottles containing sterile soilrite moistened with one-fourth strength of MS macro salts. These bottles were kept in greenhouse at 27 ± 2ºC and a relative humidity of 70 - 80%. The bottles were gradually uncapped. These seedlings after hardening were transferred to black polybags containing sandy soil (Fig. 8).



Figs. 1-8: Regeneration of plants through somatic embryogenesis. 1. Callus induction from leaflet explants. 2. Regenerative callus. 3. Globular somatic embryos. 4. High frequency of heart shaped somatic embryo. 5. Excised somatic embryos during germination. 6 and 7. Germination of somatic embryos. 8. Cloned plantlets of *V. trilobata* raised from germination of somatic embryos.

Until now there has been no report for somatic embryogenesis for *Vigna trilobata* (L.) Verdc. Attempt was made to investigate the potential of callogenesis from different types of explant *viz.* immature leaves, stem cuttings, roots and immature seeds. Of the different explants evaluated for the establishment of cell

cultures of *V. trilobata*, the small juvenile immature leaves were found to be the most suitable.

The primary callus initiated on modified MS was found to be more beneficial as compares to MS. Since the plant is native to stressed arid conditions and thus require low nitrates (Shekhawat et al. 1993). The cultures initiated from the explants were further multiplied on the fresh media. In current protocol multiplication of embryogenic culture was possible only on low concentration of auxins. An explanation for the considerably lower growth at higher auxin concentrations with increasing culture time could be growth inhibition caused by the enrichment of 2,4-D in the tissues, as was shown in *Arabidopsis* (Meijer et al. 1999).

The regenerated somatic embryos developed as new individuals that consisted in the globular stage of the typical small cells with a dense cytoplasm and developed to clear bipolar structures. The results represent a comprehensive survey of these responses, which were obtained with a small sample size and without a replication, but with a high number of growth regulator combinations. This special statistical approach is suitable to get such a survey but prevents to make differences between single treatments statistically evident. This has to be considered when such special differences will be assessed. Concerning the growth of the cultures, it became apparent that growth at a combination of 0.5 mg/l Kn and 0.5 mg/l GA_3 was found to be the optimum concentration for the regeneration of somatic embryos. Furthermore, the maximum of fresh mass increase shifted with subsequent subcultures from 0.5 mg/l Kn combined with 0.5 mg/l GA_3. 2,4-D was not required at all.

Our results show that, after regular subcultures, embryogenic responses are rarely or no longer detectable if the cultures are transferred to growth regulator free medium. Several explanations for this effect are possible. One possibility is that the medium without growth regulators might be unsuitable for the development of embryos. However, Marsolais et al. (1991) showed that the used cultivar regenerates embryos after a 2,4-D application of only two - eight days followed by a transfer to growth regulator free medium. Therefore, this explanation can be excluded. Another possibility is the embryos originate from already embryogenic determined cells. Such embryogenesis can lead directly to globular embryos through which the stock of embryogenic cells would be exhausted after a certain time. In the histological analysis of Wilson et al. (1994), no pre-embryogenic determined cells were reported. The analysis shows that normal sub epidermal parenchyma cells are the origin of embryogenesis. These cells are stimulated to embryogenesis by the application of 2,4-D. For this reason, the greatly diminished embryogenic response in a growth regulator free subculture following a longer application of 2,4-D cannot be explained by the

exhaustion of a stock of embryogenic determined cells. It has to be interpreted as an inhibition effect of the long lasting influence of the growth regulators used. This explanation is also supported by the fact that, in the case of very long subculture on growth regulator free medium, some embryos are again formed on newly formed callus. The same processes as were discussed with regard to the inhibition of growth supposedly cause this inhibition.

Present work demonstrates for the first time that it is possible to propagate embryogenic cultures in *V. trilobata* and to subsequently initiate the differentiation of embryos. However, the propagation of cells, as well as the differentiation of embryos, was inhibited for a long time by a continuous propagation on media containing 2,4-D. This knowledge could contribute to improving the propagation of embryogenic cultures and the subsequent regeneration of embryos in other *Vigna* species. One approach for such improvements might be, for example, the elimination of growth regulators after a certain time by binding them to active charcoal or washing them away by frequently changing the medium. This has proven successful in overcoming the inhibition of somatic embryogenesis caused by accumulated 2,4-D in *Arabidopsis* cell suspensions (Meijer et al. 1999). Other approaches might be the repeated switching between development and disorganization of somatic embryos, as was found suitable for maintaining embryogenic competence in carrots (Nomura 2003), or the substitution of growth regulators during the induction of somatic embryogenesis by alternative means such as salt stress, as was also possible in ginseng (Choi et al. 1998). The results of the present investigation demonstrated the establishment of a reliable *in vitro* regeneration protocol for *Vigna trilobata.*

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