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Full Length Research Paper

Abnormal plantlets regeneration through direct somatic embryogenesis on immature seeds of *Vinca herbacea* Waldst. and Kit

Babak Delnavaz Hashemloian[1]*, Azra Ataei-Azimi[1], Ahmad Majd[2] and Hassan Ebrahimzadeh[3]

[1]Biological Department, Islamic Azad University of Saveh Branch, Iran.
[2]Biological Department, Teacher Training of Tehran, Iran.
[3]Biological Department, Tehran University, Iran.

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A protocol of plant regeneration through direct somatic embryogenesis was established for the first time on *Vinca herbacea* using immature seed explants. Frequency of embryogenesis was significantly influenced by size of the seeds and growth regulators supplemented to the medium. Seeds isolated from the immature fruits between 15 and 20 days after flowering were superior in the induction of somatic embryos. Only 35% of seeds induced somatic embryos. Somatic embryos developed best on N2 medium with 0.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kin. An increase in competence for somatic embryogenesis was found with the cotyledons, while the hypocotyls part completely lost their competence. The minor seeds never initiated somatic embryo, even after 2 months of culture. Somatic embryo formation principally occurred with the major seeds. Maturation embryos developed into plantlets at a frequency of 100% when planted in free MS medium for a further 5 - 6 week period. After 5 or 6 weeks, plantlets developed in small plants. The growth rates, genotype and morphological characteristics of plantlets were different but 55% of the embryos have normal shape and 45% were abnormal.

Key words: Embryo formation, growth regulators, plantlet.

INTRODUCTION

Iranian periwinkle (*Vinca herbacea* Waldst. and Kit.) is in the Apocynaceae family, which is composed of dicots, usually herbs that have two either opposite or whorled entire leaves (Figure 1), (en.wikipedia.org/wiki/Vinca_herbacea). This plant has many alkaloid kinds with high amount (Ebrahimzadeh et al., 1996). Herbadine, a derivative of aimaline, a novel dihydroindole alkaloid, was isolated from the arterial parts of *Vinca herbaceae* (*V.libanotica Zucc*). Vincoline, an alkaloid of undetermined structure, previously isolated from *Catharanthus roseus, Vinca herbaceae*, has been reisolated and its structure elucidated (Pyuskyulev et al., 1967; Aynilian et al., 1973; Aynilian et al., 1974a,b).

Thorpe (1988) defines somatic embryogenesis (SE) as "the development of haploid or diploid cells into different-

tiated plants through embryo stages without the fusion of gametes". Ammirato (1983) gives a comparable definition. Somatic embryos are bipolar structures arising from sporophytic cells that have no vascular connection with the maternal tissue. *In vitro* somatic embryogenesis can occur from different types of explants and can be induced by the auxin analogue 2,4-D in many different species and the auxin response is quite complex (McKersie and Brown, 1996). Before somatic embryogenesis formation can commence, the presence of somatic cells capable of “receiving a command” for a switch to embryo differentiation is a prerequisite. This is

*Corresponding author. E-mail: Delnavaz@iau-saveh.ac.ir.

Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; Kin, kinetin; MS, Murashige and Skoog; 2,4,5-T, 2,4,5-threechlorophenoxy acetic acid; IAA, indole acetic acid; IBA, indole buteric acid.

Figure 1. Immature seeds of *V. herbacea* cultured in N1 at 25°C.

described as the phase of competence acquisition preceding the determination for a particular morphogenesis pathway (Christianson and Warnic, 1985). The recalcitrance of explants to the induction treatment can be attributed to an absence of competent cells and/or to the failure of cells to become competent.

Induction of somatic embryogenesis requires a change in the fate of a vegetative (somatic) cell. In most cases, an inductive treatment is required to initiate cell division and establish a new polarity in the somatic cell. In alfalfa, the inductive treatment is most commonly 2,4-D but other auxins such as 2,4,5-T are effective. The auxin response is quite complex (McKersie and Brown, 1996). Some auxins such as IAA and IBA are ineffective, and still others will stimulate the formation of embryos and callus but not somatic embryos (Shetty and McKersie, 1993). Synthetic or artificial seeds have been defined as somatic embryos engineered for use in the commercial propagation of plants (Gray and Purohit, 1991; Redenbaugh et al., 1991; Redenbaugh 1993). Various forms of synthetic seeds have been envisioned over time. The first were simply hy-drated somatic embryos produced from vegetative cells in plant tissue culture (Figure 1).

The process of production of Iranian periwinkle (*V. herbacea*) somatic embryos consists of several steps, which include the initiation of embryogenic immature seeds, the development and maturation of the embryos, and their conversion into plants. The studies conducted so far dealt only with some issues related with of *V. herbacea*. In this paper, a protocol of plant regeneration through direct somatic embryogenesis was established for the first time for *V. herbacea* using immature seed explants. An additional objective of this study was to investigate the effect of the growth regulators and seed size on the formation of *V. herbacea* somatic embryogenesis.

MATERIALS AND METHODS

Culture conditions for initiation of embryogenesis

The initiation medium was MS medium (Murashige and Skoog, 1962) with slight modifications on NH_4NO_3 and KNO_3 concentrations as described by Ramarosandratana and Van staden (2003). The medium was supplemented with 3% (w/v) sucrose and 1.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg l^{-1} kinetine (kin) (N1 medium) or 0. 5 mg l^{-1} 2,4- D and 0.5 mg l^{-1} kin (N2 medium). The pH was adjusted to 5.8, and 3 gl^{-1} of gellan gum was added to the medium prior to sterilization at 121°C, 1.05 kg cm^{-2} for 20 min.

Plant material

Iranian periwinkle (Vinca herbacea Waldst. and Kit.) seeds were collected from Siah bisheh zone (Iran) in 2006. Immature seed isolated from the immature fruit (15 - 20 days after flowering) were used as explants. Immature seeds were surface-decontaminated with 30% H_2O_2, rinsed, and then left for 12 h to imbibe prior to culture.

To evaluate the effect of developmental stage on somatic embryo initiation, immature seeds were cultured horizontally on initiation medium (N1 medium) for 2 month. The germination period was performed at 25±2°C. Thereafter, the experiment was first performed with N1 medium, and subsequently, repeated 2 month later with N2 medium. During seed germination, embryo were separated from seeds cover and placed on the initiation medium. Immature seeds cultures were kept for 2 months at 25°C in lightness (25 µ mol m^{-2} s^{-1}) without sub culturing.

Immature seeds were considered as embryogenic because 35% of the seeds initiated embryogenesis. The formation of embryogenesis was examined weekly with a binocular microscope during the second month of culture.

The embryogenic calluses and somatic embryo which developed was collected and transplanted on to a hormone-free MS medium for 2 month in test tubes and allowed to grow into small seedlings. The seedlings were then transferred to a vermiculite substrate and allowed to grow under moisture conditions.

RESULT AND DISCUSSION

Germination and growth of immature seeds

Culturing the immature seeds at 25°C in N1 medium strongly inhibited their germination for three week. In contrast, all seeds turned green when cultured at 25°C after two week. 35% of culturing the immature seeds at 25°C in N1 medium germinated after 25 days (Figure 1).

The evolution of embryogenic capacity

The effect of two combinations of plant growth regulators, N1 and N2, on the frequency of immature seeds able to form somatic embryo is presented in Table 1. The percentage of genotypes initiating somatic embryos was

Table 1. Effect of two initiation medium N1 and N2 on the percentage of immature seeds of initiating somatic embryogenesis (SE).

Medium	Somatic embryogensis (%)		
	31 days	38 days	46 days
N1	16.7	14.7	12.3
N2	36.9	28.3	20.0

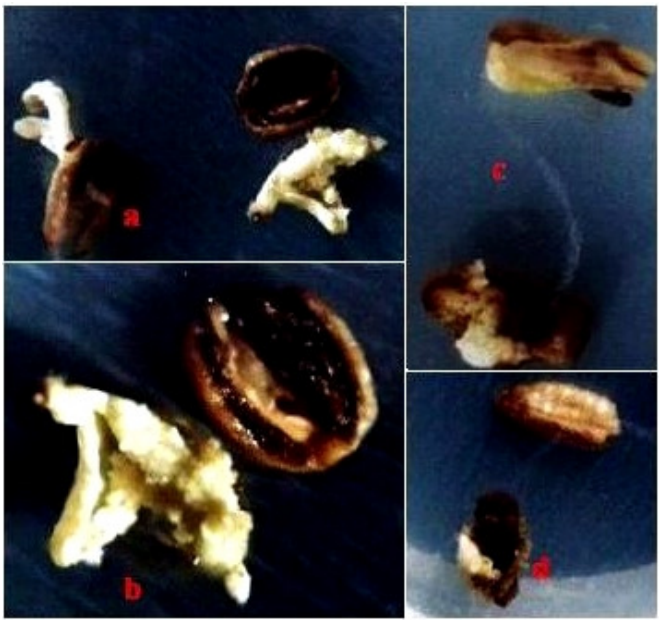

Figure 2. Immature seed germination and embryogenesis in N2 medium: **a** and **b** - major seed, **c** - medium seeds and **d** - minor seeds.

generally similar between the two media, except for the N1 was lesser than N2. In this case, N2 medium was better than N1. Somatic embryogenesis was zero after 52 day in two media (Figure 2).

Variation of the embryogenic capacity of the seeds size

The minor seeds never initiated somatic embryo, even after 2 months of culture. Somatic embryo formation principally occurred with the major seeds. The number of major seeds per all seeds ranged from 5 to 10, with an average of 36.7 major seeds per 100 seeds. During 31-46 days, major seeds somatic embryogenesis capacity of the three developmental stages was different. Somatic embryogenesis was high after 31 days but it reduced after 38 and 46 days (Table 2).

An increase in competence for somatic embryogenesis was found with the cotyledons, while the hypocotyls part completely lost their competence. These changes in competence were not dependent on physical contact between plant parts, and could not be correlated to the

Table 2. Embryogenic competence of immature seeds size at different developmental stage.

Medium	DS	Somatic embryogensis (%)		
		Minor	Medium	Major
N1	31 days	0	6.8	42.7
	38 days	0	9.3	34.2
	46 days	0	6.7	31.7
N2	31 days	0	14.2	96.2
	38 days	0	14.2	70.8
	46 days	0	12.5	48.7

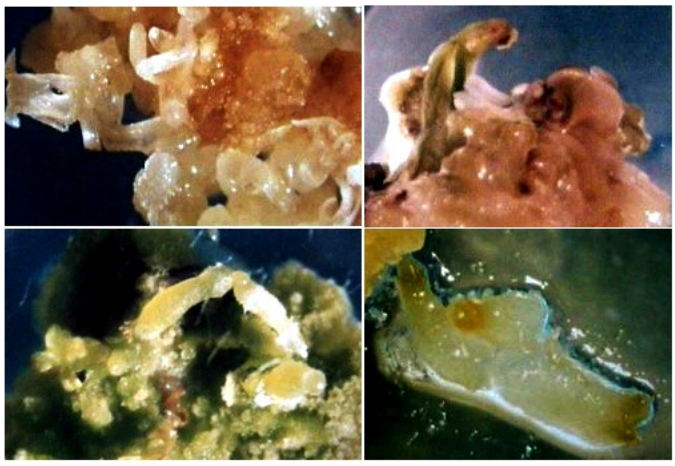

Figure 3. The major seed embryogenesis stages.

developmental stage of each part. The increase of competence in surface of cotyledon part was due not only to an increase of genotypes initiating somatic embryos, but also to an increase in embryogenicity of cotyledons.

Embryo development and maturation

The outer region of the seed embryo cotyledons explants exhibited high embryogenesis. Though the embryos developed up to early cotyledonary stage on the embryo initiation medium, they undergo conversion into plantlets. Embryos were passed from globular to cotyledonary through heart and torpedo stage (Figure 3). However, torpedo embryos were more in number. Cotyledons of some of the embryos were small and in certain cases they were unequal. Initially the cotyledons were white in colour, but later became pale green in colour. Slight callusing also occurred along with embryo proliferation. At approximately 14 days after culturing, green globular somatic embryos appear, enlarge, and develop through the heart stage into torpedo shaped embryos by day 7 - 10. Once at the torpedo stage, somatic embryos in culture typically germinate into a plantlet. These results were common with report of Anandarajah and McKersie

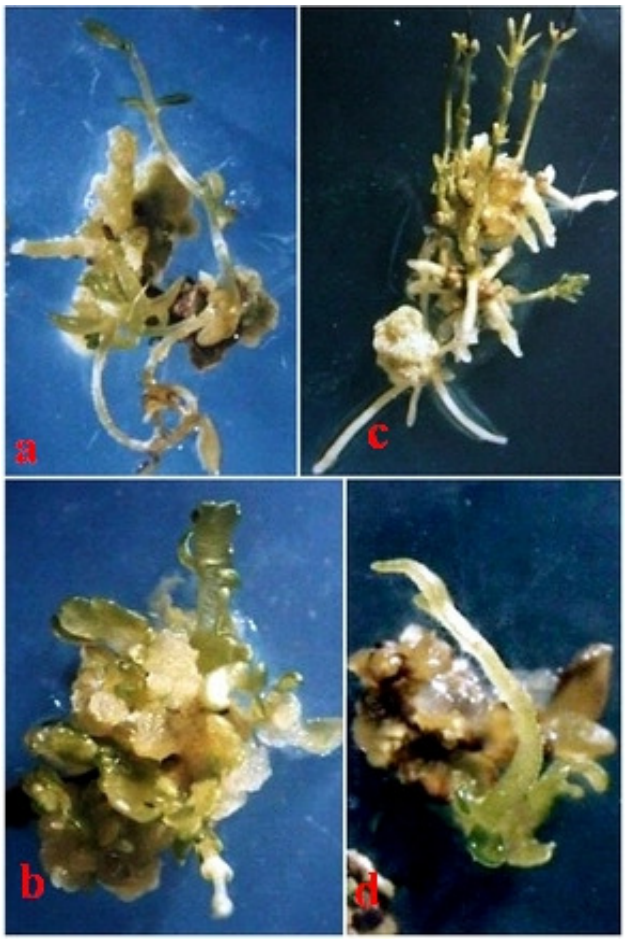

Figure 4. The first stage from different plantlets: **a** - The normal shape plantlets with two leaf in any node, **b** - succulent shapes plantlets, **c** - plantlets with 3 leaves in any nod and **d** - normal shape plantlets with one leave in any node.

(1990b). After the second sub culturing, using N1 and N2 media, at 4 week intervals, minor embryos developed to maturation embryos (Figure 3).

Variation of the somatic embryos genotype and shape

Maturation embryos developed into plantlets at a frequency of 100% when planted in free MS medium for a further 5 - 6 week period. After 5 or 6 weeks, plantlets developed in small plants. The small plants were then transplanted to a vermiculite substrate and allowed to harden off under mist conditions (Azra Ataei-Azimi, unpublished data). 55% percent of the embryos were morphologically normal. The growth rates, genotype and morphological characteristics of the plantlets were different (Figures 4 and 5): 1) Most of the plants had normal shape with two leaf in any node (nature plant) and normal growth (55%). 2) Succulent shoots plantlets had

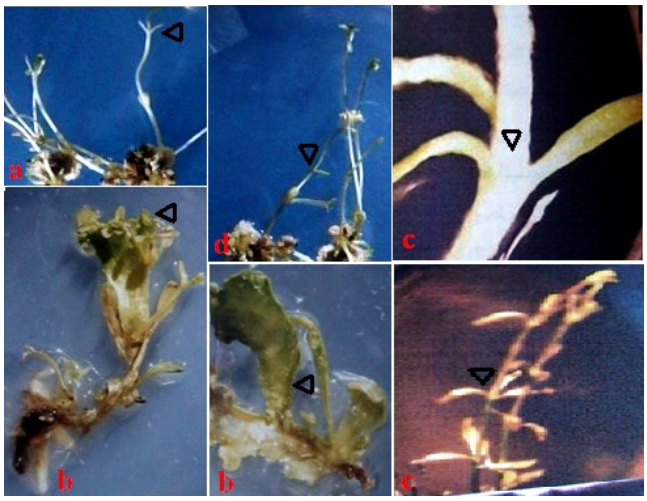

Figure 5. The secondary stage from different plantlets: **a** -The normal shape plantlets with two leaf in any node, **b** - succulent shapes plantlets, **c** - plantlets with 3 leaves in any nod and **d** - normal shape plantlets with one leave in any node.

abnormal shapes with root and small leaves, and little growth speed (33%). 3) Plantlets with 3 leaves in any nod had normal shape and normal growth (13%). 4) Normal shape with one leave in any node.

Callus was induced on the wounded immature seeds and mature zygotic embryos of *Dysosma pleiantha* (Hance) on a medium based on MS formula supplemented with 1 mg/l 2,4-D (Chuangand and Chang, 1987). This protocol of plant regeneration through direct somatic embryogenesis was established for the first time on *V. herbacea* using immature seed explants.

Conclusion

In Ataei-Azimi (unpublished data) study, the plants developed from somatic embryos seemed true-to-form which would possibly be the case even for large numbers of plants derived from seeds. However, Thorpe (1988) and Ammirato (1983) did indicate that somaclonal variation can occur but could be advantageous in the development of new plants. Rate and force of somatic embryogenesis and somaclonal variation in *V. herbaceae* is very high.

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