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Plantlet Regeneration Potential from Seedling Explants of Vitegnus (*Vitex agnus castus*)

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Abstract: In this research a simple and repeatable method for regeneration of a important medicinal plant (*Vitex agnus castus*) described. Different seedling explants such as hypocotyl, cotyledon, root and apical meristem were cultured in MS basal media with different kinds and concentrations of PGRs. Root and apical meristem explants were the only explants that have regeneration whole plantlets potential. It was interesting that regeneration whole plantlets from root and apical meristem explants have different developmental pathways. Whole plantlets from apical meristem explants regenerated by passing phase callusing whereas regeneration whole plantlets from root was direct and without phase callusing. This subject implies that we can have many manipulation possibilities in order to different objects of tissue culture by selecting different explants in vitegnus.

Key words: Medicinal plants, micropropagation, somaclonal variation, tissue culture, vitegnus

INTRODUCTION

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicines. Plants are also the source of many modern medicines. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances (Tripathi and Tripathi, 2003).

The Verbenaceae has been considered to be closely related to the Lamiaceae (Labiatae or mint family). Verbenaceae is a family of mainly tropical plants notable for heads, spikes, or clusters of small flowers. The family includes about 90 genera and nearly 2,000 species of trees, shrubs and herbs. *Vitex agnus castus* that is a important medicinal plant in Iran is a member of verbenaceae. *Vitex agnus castus* consists of the dried ripe fruits of a densely branched shrub that is indigenous to the Mediterranean and Central Asia. The commercial crops mainly come from Albania and Morocco. The plant blooms in the height of the summer and after pollination develops dark reddish brown to black fruits about the size of a pepper corn. It is this dried fruit that is used in medicinal today. This herb is probably one of the most important herbs used by Medical Herbalists to treat female hormonal disorders and is considered to be a hormonal modulator (Brown, 1994). Some of usages of this important medicinal plant are Pre-Menstrual Syndrome (PMS) (Atmaca *et al*., 2003), abnormal menstrual cycle (Bergmann *et al*., 2000) breast feeding, infertility (Cahill *et al*., 1995), hyperprolactinemia (Wuttke *et al*., 2003) and (Gorkow *et al*., 2002) and menopause (Chopin Lucks, 2003). Extract of *vitex agnus castus* as edible drop to be sold in Iran pharmacies in the name of vitegnus. Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants as bioreactors (Tripathi and Tripathi, 2003). On the other hand the fast production of a great number of genetically identical plants from a highly valuable mother plant (such as vitegnus) as one of the aim of biotechnological techniques is very important (Neumann, 1995). Also tissue culture of medicine plant is achieved in order to insure the protection of plants such as *Peperomia tetraphylla* in its natural habitat (White *et al*., 2007) or conserve valuable germplasms such as *Centella asiatica* L. (Paramageetham *et al*., 2004). The aim of this study is production of a simple and repeatable method for *in vitro* regeneration of *Vitex agnus castus* for different applications (conserve desirable characteristic and somaclonal variation).

MATERIALS AND METHODS

This study conducted in Iranian Research Institute of plant protection, Tehran, Iran in May 2005-July 2006.

Seed germination and explants preparation: Mature seeds of *Vitex agnus castus* obtained from Research Institute of Forests and Rangelands. After removal seeds coat by mechanical treatment, seeds were surface sterilized in sodium hypochlorite one quarter dilution

(5% available chlorine) for 20 min followed by 4-5 times rinses with sterile distilled water and then cultured in MS (Murashig and Skoog, 1962) medium without PGRs under 16 photoperiod at 200 μm mol^{-2} sec^{-1}. Hypocotyl (3-4 mm), cotyledons (2-3 mm), root (2-3 mm) and apical meristems were excised from 10-days-old aseptically grown seedlings for preparation of explants.

Media and culture conditions: The basal medium tested in these experiments was MS. All of media containing 3% sucrose and 0.9% agar and supplemented with combinations of 0.4-1.5 mg L^{-1} IAA plus 0.2-2 and 1 mg L^{-1} NAA plus 0.5 mg L^{-1} KN. MS medium free from PGRs was also used as control. All of media adjusted to pH 5.8 prior to autoclaving at 120°C for 20 min. All cultures incubated at 24±2°C in a incubator under dark conditions for callus induction and growth of roots and 200 μm mol^{-2} sec^{-1} for growth of shoot and plantlet regeneration.

Experimental design, data collection and analysis: Experiments were set up in Completely Randomized Design and repeated three times. Each treatment has 10 replications. Observation on the number of explants that produced whole plantlet were recorded. Data were subjected to SD and ANOVA test.

RESULTS

The seeds with intact seed coats did not germinate, even after 14 days. After mechanical treatment (scrape of seed coat) seeds germinated easily after 3-4 days.

Effect of different explants on plantlet regeneration Hypocotyl: After 2-3 weeks of culture, hypocotyls explants produced white calli and after this stage (10-20 days later) white calli differentiated to roots. Calli and regenerated roots usually were originated from two ends of explants (Fig. 1a) Also hypocotyls explants in medium with different kind of auxine and cytokinine had different reactions. These reactions were different and have different forms such as shooting with phase callusing and without of it (very short phase callusing) and shooting with rooting (Fig. 1b-d).

Cotyledon: Cotyledon explants in different kind but equal balance of auxine and cytokinine (1 mg L^{-1} NAA plus 0.5 mg L^{-1} KN and 1 mg L^{-1} IAA plus 0.5 mg L^{-1} BA) produced white calli and compact calli that differentiated to roots (Fig. 1e).

Root: The first reaction of root explants after establishment of them in medium with 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN was growth and multiple of it so that the one very small pieces of root (2 mm) converted to very long and thick roots with a lot of lateral roots (Fig. 1f).

The other response of root explants in medium was production of regenerated leaves. Not only root explants but also regenerated roots that obtained from culture of cotyledon in medium with 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN produced regenerated leaves (Fig. 1g and h, respectively).

Root explants in above medium (1 mg L^{-1} NAA and 0.5 mg L^{-1} KN) had also planlet regeneration potential. In fact the most important response of root explants in media was production of regenerated whole plantlet (Fig. 1i).

Apical meristem: Apical meristem explant in different media exhibited different responses, so that in media with 1 mg L^{-1} IAA and 0.5 mg L^{-1} BA and 0.4 mg L^{-1} IAA plus 0.2 mg L^{-1} BA response of it was regeneration of shoots. Regeneration of shoots from apical meristem explants was with production of cotyledonary leaves (Fig. 1j and k, respectively).

In medium with 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN apical meristem explants produced small calli at first and after a little growth of these calli signs of differentiation of regenerated shoots and roots were appeared in calli and similar to root explants, apical meristem explants had also potential of regenerated plantlets (Fig. 1l) but with high percentage than explants root (87.54±0.9772 and 79.93±1.009, respectively).

DISCUSSION

Intact seeds of *Vitex agnus castus* without mechanical treatment did not able to germination. It is due to the seed coat may be acting as a barrier to water and or gas permeability and intact seeds may have been still in a state of dormancy which was only broken in the culture environment after the removal of the seed coat (Mao, 1995).

The basal media used in this research was MS, because present findings and other researchers showed that not only in verbenaceae (Sunitibala Davi *et al*., 1994; Varma *et al*., 1992; Yang *et al*., 1992), but also in other families of plants (Caboni *et al*., 2000; Prakash *et al*., 2004; Onay *et al*., 2004; Wang *et al*., 2004; Majd *et al*., 2006) MS basal medium is one of the best basal media for different purposes of tissue culture.

The reason of callusing and then root regeneration and or growth of it in presence of 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN is clear. Cytokinines are one of the most important factors for division of cells, on the other hand NAA is the one of the best PGRs for callusing

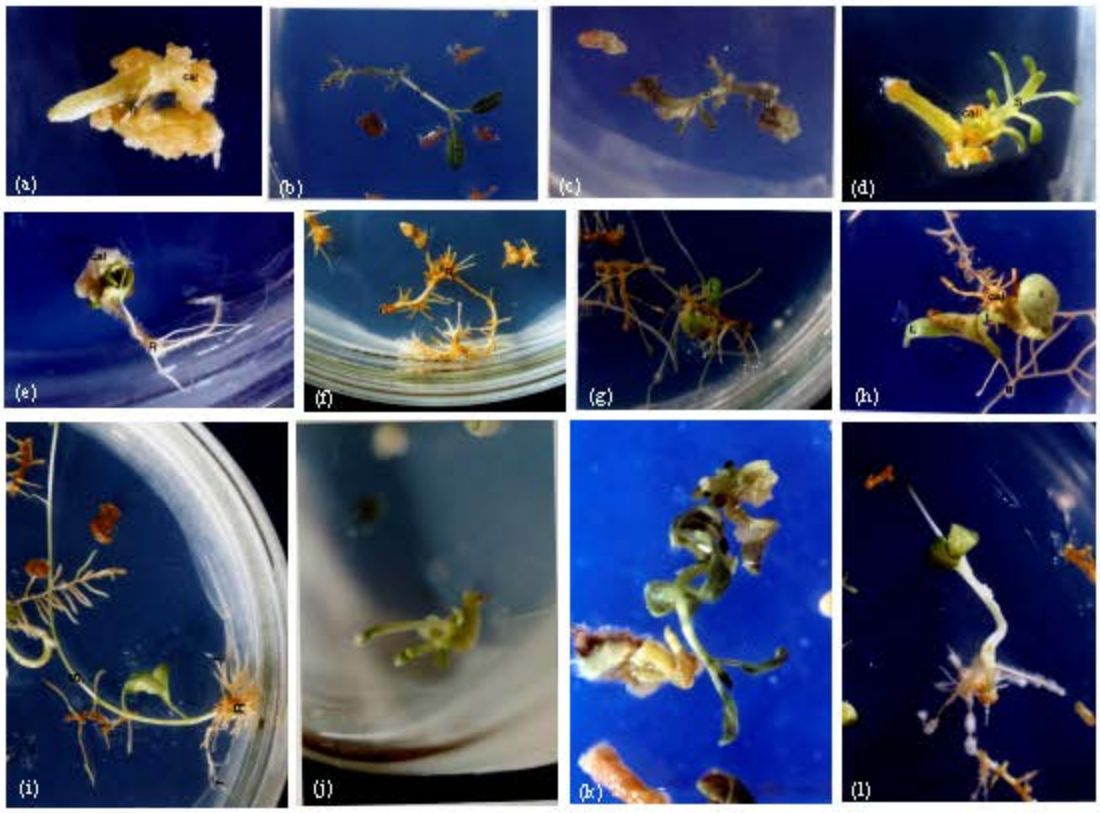

Fig. 1: Effect of different kinds and concentrations of PGRs on seedling explants of vitegnus (*Vitex agnus castus*). (a) Callusing and rooting on hypocotyls in 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN, (b) Effect of 1 mg L^{-1} BA and 0.5 mg L^{-1} IAA on young stem, (c) Effect of 1 mg L^{-1} IAA and 0.5 mg L^{-1} BA on young stem explants, (d) Effect of 2 mg L^{-1} BA and 1.5 mg L^{-1} IAA on young stem, (e) Callusing and rooting on cotyledons in 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN, (f) Effect of 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN on root explants, (g) Regeneration of leaves on root explants in 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN, (h) Callusing and rooting on cotyledon explants and after regeneration of leaves from regenerated roots, (i) Regeneration of whole plantlet from root explants in 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN, (j) Regeneration of leaflet stem on apical meristem explant in 1 mg L^{-1} IAA and 0.5 mg L^{-1} BA, (k) Regeneration of leaflet stem on apical meristem explant in 0.4 mg L^{-1} IAA and 0.2 mg L^{-1} KN and (l) Callus and whole plantlet from apical meristem explant in 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN

(Remotti and Hubertus, 1995). In this medium hormonal balance is suitable for rooting (auxine higher than cytokinine). Varma *et al*. (1992) explained that the best medium for growth of callus of vitex is 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN that this finding is agreement to our finding. There are other reports that show in rate hormonal opposite above medium (1 mg L^{-1} KN and 0.5 mg L^{-1} NAA) at first callusing then rooting were occurred such as *Digitalis thapsi* (Tarrago *et al*., 1990). The reason of this apparently opposite findings can be: (a) Inter hormonal contain of plants (Centeno and Berros, 1997) and (b) Vigour of NAA as a artificial auxine.

Based on our experience in this study, in different media with rates of BA in all cases shooting were seen. This implies the importance of BA in regeneration of shoot.

Bergman and Monn (1997) reported that not only BA is necessary for shooting but also the number of regenerated of shoots related to level of BA in media in *Paulownia elongeta* and *Paulownia fortunei*. The reports of Caboni *et al*. (2000), Tang *et al*. (2004) and Chamandoosti *et al*. (2006) in apple, pinus and canola respectively justifiy present results for importance of BA in vitegnus.

Apical meristem and root explants in medium with 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN produced whole plantles. At first this subject must be concerned that culture of apical meristem explants for micropropagation of plants is very current in tissue culture systems (Tarrago, 1990).

Although the shoot apical meristem explants are a population of cells located at the tip of the shoot axis and they produces lateral organ, stem tissues and regenerates itself, but in culture media shoot apical meristem explants produced only shoot and also meristematic cells of root meristem explants produced root, on the other hand regeneration of root from shoot meristem and shoot from root meristem began with callusing (Sachs, 1984) therefor regeneration of whole plant with culture of apical meristem and short phase callusing in our experience is completely usual.

Root explants similar to apical meristem explants had potential of regeneration plantlets, in medium with 1 mg L^{-1} NAA and 0.5 mg L^{-1} KN, but without passing of phase callusing. Muthukumar *et al*. (1995) and Xiao *et al*. (1995) reported also regeneration of plantlets from root explants.

Regeneration of plantlets from root and apical meristem explants in media with equal kind and concentration of PGRs (1 mg L^{-1} NAA and 0.5 mg L^{-1} KN) is very interesting results of this study.

Tissue culture systems have many interseting and different applications for plant science biologists, such as somaclonal variation and micropropagation. Somaclonal variation that basis of it is rapid cell divisions *in vitro* when occurred that regenerated organs (root and shoot) and plantlets were produced by passing from interphase callusing (Tarrago *et al*., 1990) so that some scientists believe that some form of somaclonal variation may result from mechanisms that also occur in somatic tissue *in vivo*, cultured cells may show an accelerated and perhaps controllable rate of variation (Lee and Phillips, 1988). On the other hand the purpose of micropropagation is rapid clonal of plants in order to saving and increasing of desirable characteristic of plants. So regeneration vitex plantlets from apical meristem and root explants is useful for two different purposes (somaclonal variation and micropropagation) and give us many possibilities for manipulating in *in vitro* culture of vitegnus.

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