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Improved effect of glutathione on the induction and growth of *Taxus baccata* L. callus

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ABSTRACT

Yew (Taxus baccata. L) as a tree species native to Iran, has always been the center of attention due to the high amount of active components. One of these compounds is taxol (paclitaxel) which is effective in treatment of variety of tumors and cancers. Nowadays numerous advanced biotechnological methods such as tissue culture are in progress in order to increase the amount of compounds. In this study, to produce callus, both stem and leaf explants were cultured in vitro under different treatments in order to select the optimal medium condition in which better quality callus was produced in shorter time. Stem explants produced better callus and B5 medium supplemented with 20 g/L sucrose, 8 g/L agar, 0.5 mg/L 1-naphtaleneacetic acid, 0.4 mg/L kinetin, 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.1 mM gluthatione and 500mg/L Casein was the more effective medium. To reduce tissue browning different antioxidants were tested and 0.1 mM gluthatione was selected as an effective agent that considerably reduced tissue browning process and also stimulated the callogenesis and growth of callus.

Key words: *Taxus baccata*, tissue culture, gluthatione, browning.

INTRODUCTION

Plant tissue culture refers to growing and multiplication of cells, tissue and organs of plant on defined solid or liquid media under aseptic and controlled environment. *Taxus baccata* (Yew tree), a medicinal and protected plant, is distributed in north Asia, Europe and some northern part of Africa. *Taxus* produces a class of natural compounds known as taxane diterpenoids or taxoids characterized by the taxane skeleton. The most economically and pharmaceutically important of the compounds is the anti-cancer drug, paclitaxel, known as taxol. This compound was approved to have clinical treatment of ovarian and breast cancer by the United States Food and Drug Administration (FDA). In addition, taxol has significant activity in the treatment of malignant melanoma, lung cancer, and other solid tumors [10,11]. But *Taxus* species grow very slowly and require long time of seed dormancy which is up to 1.5 to 2 years [8] and the extraction of paclitaxel from naturally growing *Taxus* trees is quite limited because a large number of trees need to be harvested to obtain a sufficient amount of paclitaxel [21]. Several alternative sources of paclitaxel have been identified and are currently the subjects of considerable investigation worldwide. These are: the agricultural supply of taxoids from needles of *Taxus* [32]; hemisynthesis [15,12]; total synthesis [28,29,16]; prospecting of new natural source of paclitaxel, fungus production [5]; and production of taxoids by *Taxus* cell and tissue culture. This work concentrates on the latter possibility. Large-scale use of plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemicals independent of plant availability [19,27]. Tissue and cell culture of *Taxus* sp. is being considered as a very promising approach towards providing a long-term source of this valuable compound so the application of plant biotechnology techniques can be valuable in order to improve the use of *Taxus* for medicinal purposes. In this investigation we studied some factors that affect callogenesis and growth of callus. To approach

this purpose analyzing the effect of explants type and plant growth regulators on callus initiation and growth and effect of different antioxidants on browning was carried out and then the fast growing callus cultures of *Taxus baccata* were selected.

MATERIALS AND METHODS

Explants of *Taxus baccata* tree from Shahid beheshti University's garden were used as the plant material for callus induction. The explants included young stem and needles.

For callus culture the explants (stem segment and needles separately) were washed with distilled water and soap, immersed for 15 minute in 3% sodium hypochloride (MERK, Germany) then immersed for 60 second in 70% ethanol and finally were rinsed three times with sterile distilled water. Briefly, the sections of young stem and needles (length 0.5-1.0 cm) of *Taxus baccata* were prepared. For callus induction prepared explants were placed in the gamborg B5 medium [26] supplemented with sucrose (20 g/l), plant agar (8 g/l), B5 vitamins, myoinositol (100 mg/l), casein (500 mg/l) plant growth regulators include 1-naphtaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kin) with different concentration and 0.1 mM glutathione (GSH), 25mM salicylic acid (SA) and 500 and 1000 (mg/l) ascorbic acid (all from MERK Germany). The pH of the callus induction media was adjusted to 5.8 prior autoclaving. During this induction process, the cultures were kept at 25 ºC in dark condition and subcultures of calli were done every two week.

RESULTS AND DISCUSSION

Effects of explant type on callus formation

Callus tissues from young stems were induced better than from needles (Fig 1A, 1B). The slow growth rate of callus initiation and growth was observed for all needles and stems explants in the present study. This slow growth might be due to the presence of certain growth inhibitors.

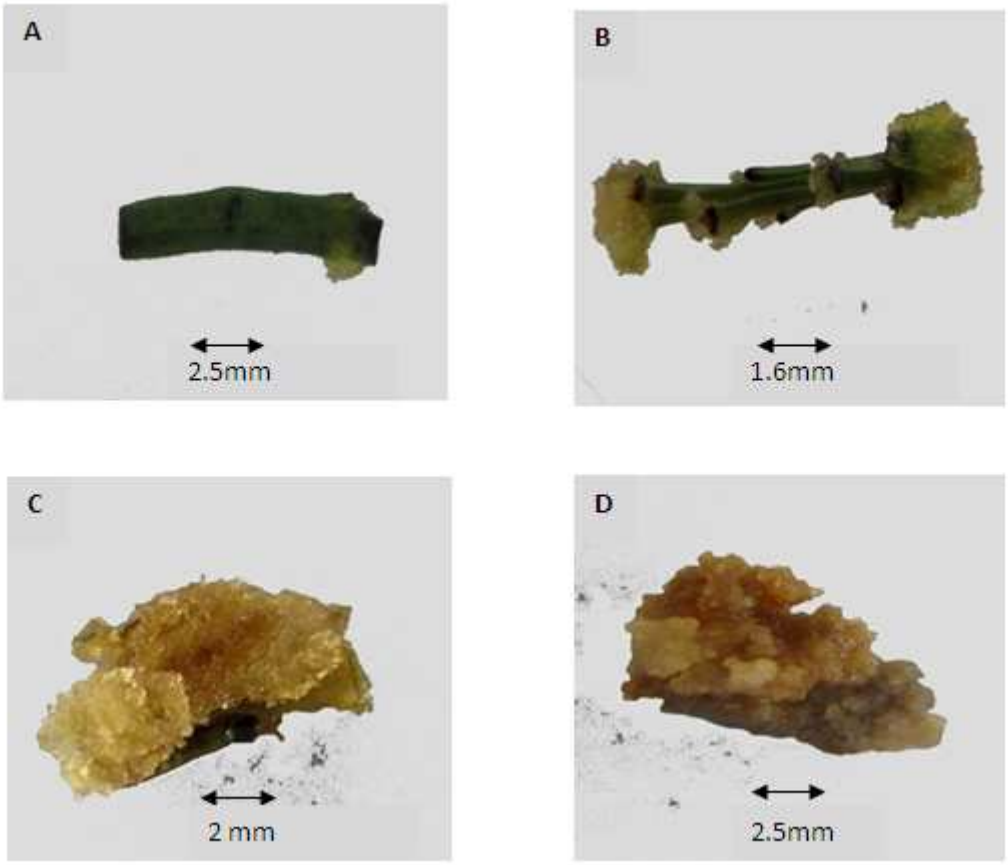

Figure1: Effect of explants source on callus formation. A: needle with small callus and very slow growth (12 days callus). B: stem with callus that its formation initiated around the cutting area (12 days callus). C (20 days) and D (30 days) growth of stem explants callus during different days.

Calli developed from the division of cambium and outer parenchyma tissue of the stem, since the first cell proliferation started on the outer layer of the explants [1,2]. And different response between stem and needles might be related to the relatively small amount of meristems and parenchyma in needles [30]. Medium components uptake more in the cutting area of young stem explants and so calli induction was initiated around this area. In our experiment at first, small-sized calli were observed at the edge of explants after about 12 days of culture (Fig 1B) that started sooner in comparison with later studies.

Effect of plant growth regulators on callogenesis

To analysis the effect of plant growth regulators different combinations at different concentrations were tested (Fig 2). Plant hormones were tested in 4 individual experiments.

Auxin induced the formation of callus. 2,4-D was the most effective in callus formation. Alternatively the combination of auxin and cytokinin enhances the callus induction. This results obtained after 30 days of culture (Figure 2). Calli were observed on all the media used in this experiment. But rapidly browning of the explants is a real problem in *T.baccata* callus culture especially during the first 2 weeks of culture.

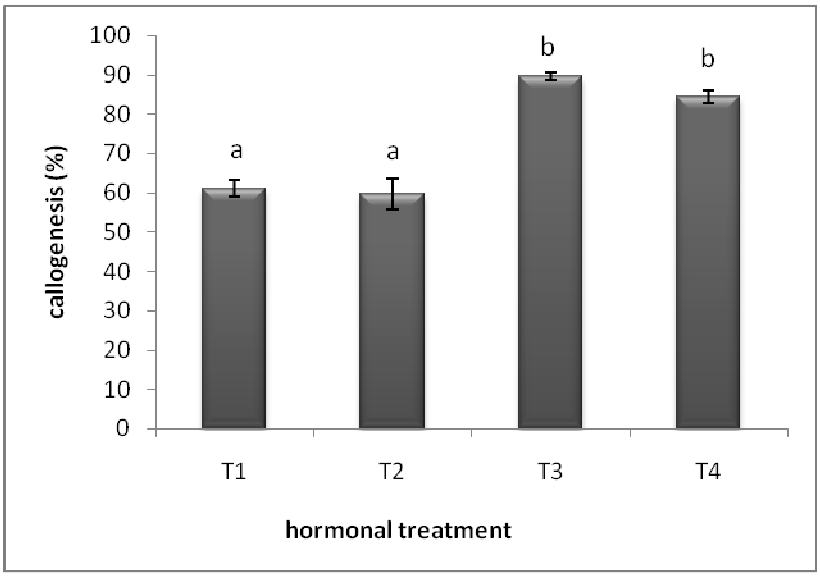

Figure2: Effect of PGR on *T.baccata* callogenesis.

T_1: NAA 2mg/L ,Kin 0.4 mg/L ,2,4-D 0.5 mg/L ,GSH 0.1 mM ,Casein 500mg/L
T_2: NAA 2mg/L ,Kin 0.4 mg/L ,2,4-D 2 mg/L ,GSH 0.1 mM ,Casein 500mg/L
T_3: NAA 0.5mg/L ,Kin 0.4 mg/L ,2,4-D 0.5 mg/L ,GSH 0.1 mM ,Casein 500mg/L
T_4: NAA 0.5mg/L ,Kin 0.4 mg/L ,2,4-D 2 mg/L ,GSH 0.1 mM ,Casein 500mg/L
Data are the mean of 3 repeats of 100 explants (±SE)

Browning

This process is a result of the production and oxidation of phenolic compounds by the explants. Browning was frequently encountered during initial stages of culture and eventually lead to the death of the tissue if the production of polyphenols, resulting of defense reactions is excessive. Kim et al (2005) [6] has also observed browning in *Taxus* sp. culture that caused cell growth reduction and it appears to be related causally to taxol production. Secondary metabolites are often produced as part of a plants' defensive mechanism [24], and that compounds may be implicated in apoptosis and death of cells [23,34] and thus affected callus growth index. Frequent sub culturing might reduce this problem but not completely. About this problem the effects of supplements such as activated charcoal in medium were also investigated by Fett-Neto [2]. The application of activated charcoal decreased browning of callus, but also reduced the growth and development of the explants. This is possibly due to the absorption of plant growth regulators (PGR) and/or salts besides the phenolics from the medium by activated charcoal [22,31,20]. This leads to the fact that the concentration of auxin and cytokinin in the media decreased remarkably, and hence was not enough for callus inducement.

To prevent browning, we developed a method using antioxidants during early phase of culture. SA is an important signal element and endogenous growth regulator that is involved in tolerance to multiple stresses in plants [3,4].

Earlier investigations have shown the role of SA in modulating plant responses to a wide range of oxidative stress [18] and in mustard and maize exogenous application of SA increased the antioxidant efficiency that induced plant tolerance [13,14,33].

Ascorbic acid acts as an antioxidant. Oxygen preferentially reacts with the ascorbic acid, rather than the phenolic compounds in tissue. Browning does not proceed until all the ascorbic acid is used up in the reaction.

It is believed that among antioxidants the key reductant in living cells is GSH which is very abundant [25]. There is considerable evidence that GSH plays a vital role as an antioxidant in the defense systems of plants against environmental stress [7] and known to relieve stress conditions such as oxidation. Level of GSH and differentiation are closely related in plant cells. In apple culture, medium containing GSH promoted callus growth [17], and also in *Pistacia vera* shoot tip culture [9] results indicated that GSH reduced the total phenolic compounds and this treatment significantly promoted the growth.

The effect of GSH, SA and ascorbic acid on browning and growth of *T.baccata* callus culture are showed in table 1. According to these results the application of GSH in the medium considerably reduced tissue browning process to 8.61% but SA and ascorbic acid have a little effect. Also GSH initiated more rapidly callogenesis after 12 days and the callus formation rate on medium supplemented with 0.1mM GSH was 89.61% in young stem explants on B5 medium, twice of control (Table 1).

Table 1: effects of different antioxidants on callogenesis and callus browning.

Data are the mean of 3 repeats of 100 explants (±SE)

treatment	Callogenesis (%)	Browning (%)
Control	48.96± 9.1 (a)	48.96± 9.1 (a)
GSH 0.1mM	89.61± 0.96 (b)	8.61± 0.82 (b)
Asc 500mg/l	54.86± 3.01 (a)	40.69± 0.57 (a)
Asc 1000mg/l	52.62± 3.13 (a)	37.86± 1.14 (a)
SA 25mM	61.2± 0.89 (a)	33.12± 2.07 (a)

CONCLUSION

T.baccata stem explant can be cultured on B5 basal medium supplemented with antioxidant and PGRs at appropriate concentrations. Among Auxins, 2,4-D was more effective than NAA in callus initiation and development and GSH that reduced browning of tissues may be consider to improve callus growth of this species which has a particularly slow growth response *in vitro* probably due to a high oxidative stress at which tissues respond poorly without the addition of exogenous antioxidants such as glutathione.

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