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Original Article

Effects of Different Concentrations of 2, 4-D and Kinetin on Callogenesis of *Taxus Brevifolia* Nutt.

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Abstract

Taxus is an endangered plant which is the only commercial source of paclitaxel, which is used for treatment of ovary and breast cancer. As production of this valuable drug is a destructive process, other alternatives should be considered for its sustainable production. Plant tissue and cell culture is a promising method for production of secondary metabolites. In order to optimize yew callus culture effects of 16 combinations of two plant growth regulators, 2, 4 dichlorophenoxyacetic acid (0, 0.5, 1 and 1.5 mg/L) and Kinetin (0, 0.1, 0.5 and 1 mg/L) on callogenesis of *Taxus brevifolia* twig explants were studied. Considering growth criteria, fresh weight (33.5 mg), dry weight (3.88 mg) and callogenesis ratio (1.45), based on statistical analysis the best plant growth regulators combination for induction and growth of callus of twig explants was appeared to be 1.5 mg/L of 2, 4- dichlorophenoxyacetic acid and 0.1 mg/L of Kinetin.

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Introduction

Taxus is the only commercially important genus of taxaceae [1]. Increasing demand for paclitaxel to treat ovary and breast cancer at the late 90s [2-4] resulting in over-harvesting the plant resources has endangered yew plant [5]. Regarding limited resources and environment protection regulations in Iran, biotechnological approaches should be applied especially chemical synthesis of paclitaxel is not commercially viable [6-8]. Plant tissue culture is a sustainable method for production of anti-cancer metabolites [9]. Calli of *Taxus* spp. have been obtained using different tissue explants including the bark, shoot, green and red arils, seed parts, young stems and needles [9-11]. Of these, young stems were the best source of explants and the resulting calli grew fast [12]. Among the factors optimizing growth criteria the constituents, concentration and combination of plant growth regulators (PGRs), play an important role in callus induction and growth [13-15], generally the medium contained 1-2 mg/L of 2,4- dichlorophenoxyacetic acid (2, 4-D) or its combination with other PGRs [12]. Naphthaleneacetic acid (NAA), picloram and Kinetin were also essential in some cases [12, 14, 16-18] but the most effective auxin compound and concentration have varied with respect to explant tissue type [14]. At the present research combinations of 2, 4-D and Kinetin in Murashige-Skoog (MS) medium basal salts were applied to optimize PGRs concentration for callus induction and growth.

Materials and Methods

Plant sample

Four years old pacific yew plants were obtained from the ecology garden of Noshahr in north of Iran.

Medium preparation

Macronutrients, micronutrients and Fe-EDTA were prepared according to MS formulation [19] supplemented with 50 mg/L of ascorbic acid, 2 mg/L of glycine, 30 g/L of sucrose, 7 g/L of agar and PGRs. 2, 4-D and Kinetin were added according to Latin square in concentrations (0, 0.5, 1 and 1.5 mg/L of 2, 4-D) and (0, 0.1, 0, 5 and 1 mg/L of Kinetin). A total of 16 treatments of these PGRs were applied. The pH of the culture medium was adjusted to 5.8±0.05 before autoclaving for 20 min at 121°C.

In vitro culture

5-10 cm long cuttings were excised from twigs. Their leaves were removed and disinfected with 70 % ethanol (5-10 sec), washed with sterile distilled water (SDW) 1-2 min, 1 % commercial bleach (pH was adjusted to 6) for 4-5 min and finally rinsed three times with SDW (5,10 and 15 min respectively). Disinfectant exposed ends were removed and 2-3 mm explants were cut. In each treatment 24 explants, were cultured in separate 60 ml vials containing 20 ml of the medium. Vials were capped with 2 layers of aluminum foils and incubated at darkness at 24°C. Calli were subcultured after 55 days and harvested after 85 days for assaying fresh weight (FW), dry weight (DW) and callogenesis ratio (CR).

Statistical analysis

For statistical analysis CRs (explants produced callus per total explants) were changed to their arc sine. Data analysis was conducted according to factorial design using Jump software (JMP® 3.1.2, 1989-1995, SAS Institute

Inc.). Mean separation test was done using Duncan's multiple range test at alpha level 0.05 (MSTAT 1.42, 1987-1989, Knowledge Dynamics Corporation).

Results

Results of data analysis showed that both 2, 4-D and Kinetin increased FW and 2, 4-D was more effective with inductive effect on callus growth (Fig. 1). Combination of these PGRs was more effective than the application of each individuals, referring synergistic effect of 2, 4-D and Kinetin in optimum range of concentrations (0.1– 0.5 mg/L Kinetin). Further concentrations of Kinetin had inhibitory effect on growth of callus and FW (Fig. 2), while 2, 4-D showed a linear stimulatory effect on callus growth (FW) (Fig. 3).

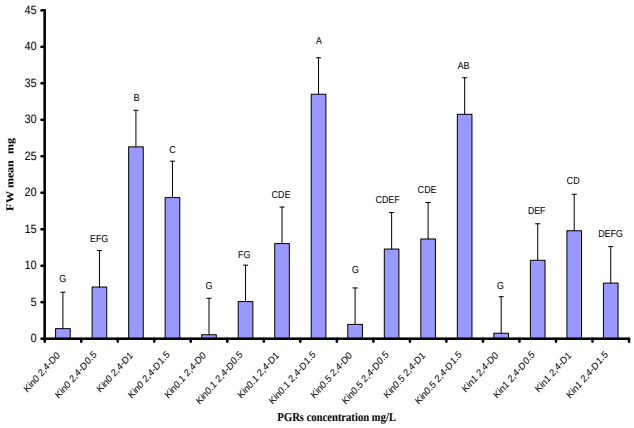

Figure 1. Relationship between FW of twig derived callus of *T.brevifolia* and PGRs combinations of 2, 4-D and Kinetin.

Among 16 treatments, hormone combinations of 1.5 mg/L 2, 4-D ; 0.1 mg/L Kinetin and 1.5 mg/L 2, 4-D ; 0.5 mg/L Kinetin appeared to be the most effective hormone combinations on the growth of callus tissue (FW and DW) but, no statistical difference was observed between the two treatments.

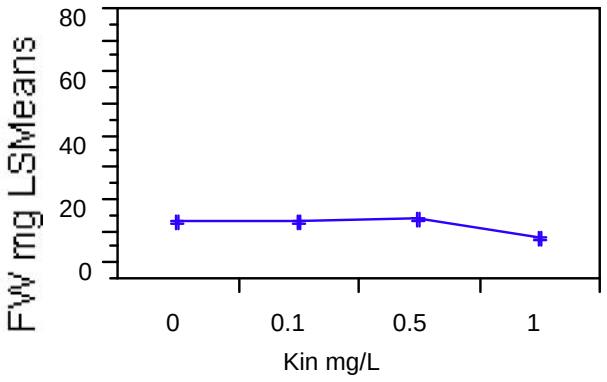

Figure 2. Relationship between FW of twig derived callus of *T.brevifolia* and Kinetin concentration.

While 2, 4-D concentrations higher than 1 mg/L still had increasing effect on DW (Fig. 4 & 5), Kinetin concentrations higher than 0.5 mg/L had inhibitory effects (Fig. 4 & 6). Although, 2, 4-D concentrations in the range of 0.5-1 mg/L had a sharp increase of callus DW, 0.00-0.5 mg/L 2, 4-D and concentration of 1.5 mg/L had a slow increasing effect on callus DW (Fig. 5) and the most effective range of Kinetin was 0.1-0.5 mg/L (Fig. 6).

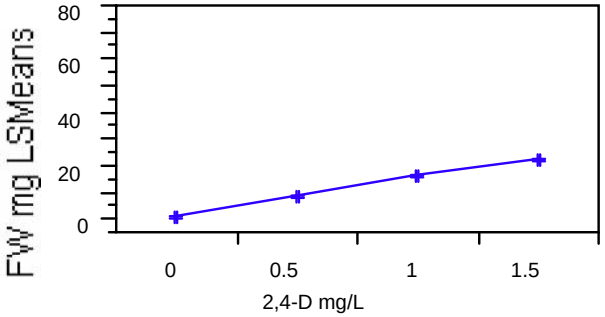

Figure 3. Relationship between FW of twig derived callus of *T.brevifolia* and 2, 4-D concentration.

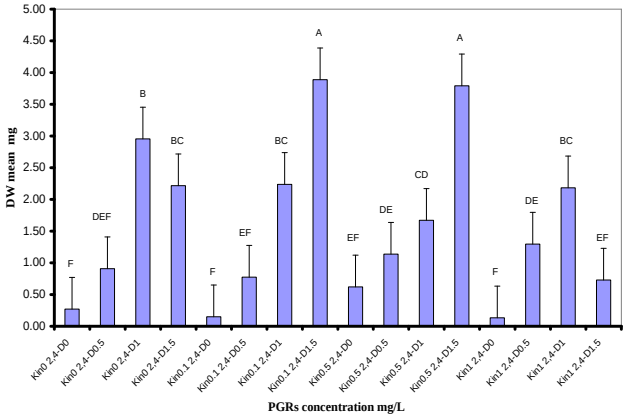

Figure 4. Relationship between DW of twig derived callus of *T.brevifolia* and PGRs combinations of 2, 4-D and Kinetin.

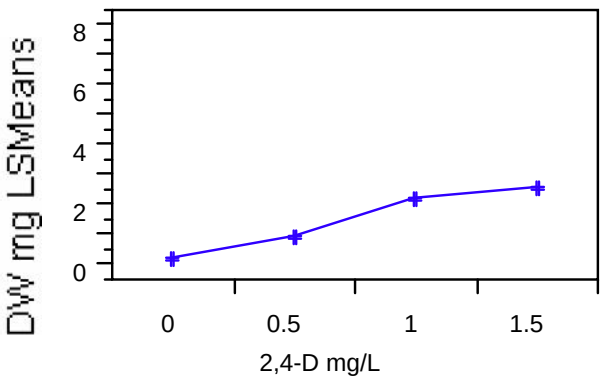

Figure 5. Relationship between DW of twig derived callus of *T.brevifolia* and 2, 4-D concentration.

Results indicated that 2, 4-D appeared to be the most effective hormone factor responsible for callogenesis (Fig. 7 & 8) but Kinetin had no increasing effect on CR (Fig. 7 & 9) especially concentrations higher than 0.5 mg/L. On the basis of the mean test it appeared that 1.5 mg/L 2, 4-D and 0.5 mg/L Kinetin was the best hormone combination for callogenesis.

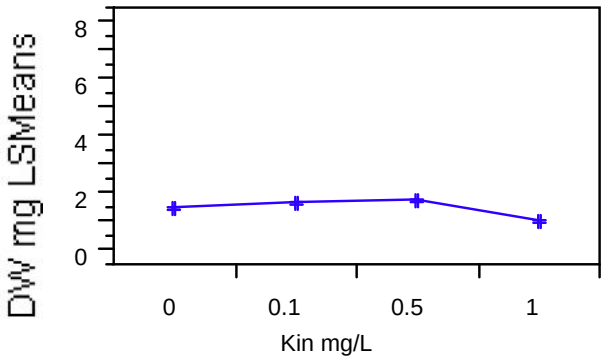

Figure 6. Relationship between DW of twig derived callus of *T.brevifolia* and and Kinetin concentration.

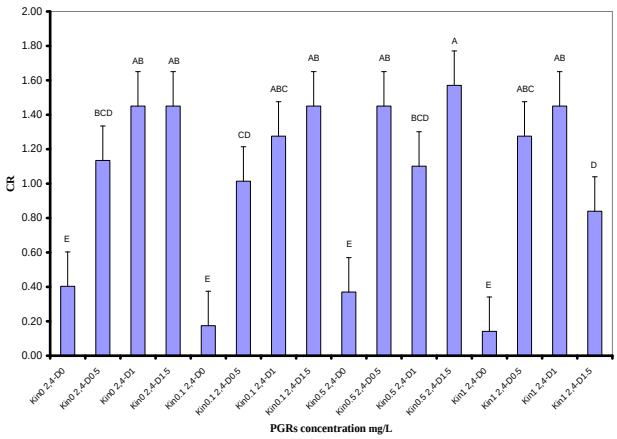

Figure 7. Relationship between CR of twig explant of *T.brevifolia* and PGRs combinations of 2, 4-D and Kinetin.

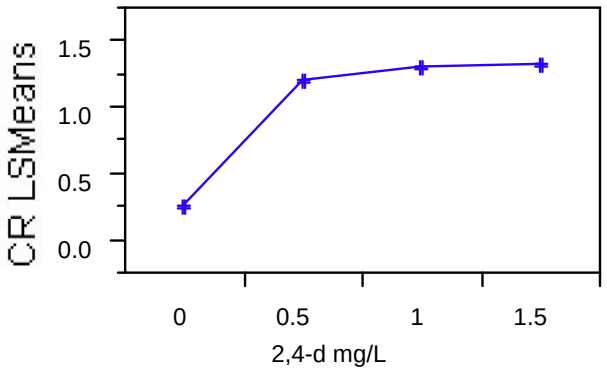

Figure 8. Relationship between CR of twig explants of *T.brevifolia* and 2, 4-D concentration.

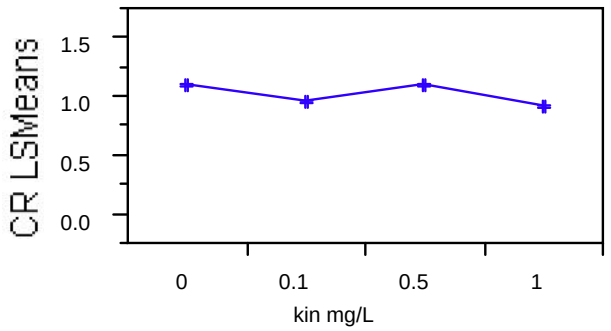

Figure 9. Relationship between CR of twig explants of *T.brevifolia* and Kinetin concentration.

Discussion

Results of effects of 16 combinations of 2, 4-D and Kinetin on FW, DW and CR of twig derived callus of *T. brevifolia* showed increasing effect of 2, 4-D application on these factors which is in agreement with prior reports [14, 20]. Although 1 mg/L 2, 4-D has been reported to be optimum concentration for callus induction using B5 medium in common yew [16] our results suggest that 1.5 mg/L 2, 4-D has increasing effect on callus induction and on FW, DW and CR using MS medium basal salts. Callus culture of young stems and needles of *T. wallichiana* were established on modified MS media supplemented with 0.00-5 mg/L 2, 4-D, 0-3 mg/L NAA and 0.00-0.25 mg/L Kinetin indicated that a medium containing 2, 4-D (5 mg/L), Kinetin (0.25 mg/L) was optimal for stem derived callus growth whereas the presence of NAA (3 mg/L), Kinetin (0.25 mg/L) in the medium induced optimal needle callus growth [18]. This difference is possible due to culture media, plant species or variety or even replicate number or different incubation conditions. In addition, using different media, different results have been obtained and 1-2 mg/L 2, 4-D alone or in combination with other PGRs has been reported as optimum concentration for callus induction and growth [12]. Concentrations of 2 , 3 and 5 mg/L 2, 4-D supplementing MS medium had been appeared to be as optimum concentrations for stem derived callus [14, 18]. Higher concentrations of 2, 4-D up to 2 mg/L or more are expected to give better results than lower concentrations.

Kinetin (0.1-0.5 mg/L) had a pronounced effect on FW and DW that agrees with previous findings [12, 16]. No significant difference was observed between the two concentrations. 0.1 mg/L Kinetin appeared to be the optimum concentration. This agrees with prior research have shown low concentrations of Kinetin have increased callogenesis but higher concentrations have been inhibitory [16]. Kinetin had no significant effect on CR and some fluctuations were observed. Callus induction effect of Kinetin is not likely and the only effect of this hormone on callus FW and DW was observed.

Combination of 2, 4-D and Kinetin was appeared to be effective on FW and DW. The best PGRs combinations were appeared to be 2, 4-D (1.5 mg/L), Kinetin (0.1 mg/L) and 2, 4-D (1.5 mg/L), Kinetin (0.5 mg/L). As there was no significant difference between the two, 2, 4-D (1.5

mg/L), Kinetin (0.1 mg/L) is suggested for tissue culture of pacific yew.

With respect to callogenesis effect of 2, 4-D and inhibitory effect of Kinetin on twig explants of yew in this experiment and other reports, 1.5 mg/L 2, 4-D or higher is recommended to be used and if Kinetin is to be used, hormone combinations should be optimized in the range of 0.1-0.5mg/L Kinetin.

Conclusions

Based on statistical analysis the best plant growth regulators combination for induction and growth of twig derived callus was appeared to be 1.5 mg/L of 2, 4-D and 0.1 mg/L of Kinetin.

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