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Full Length Research Paper

Plant regeneration through indirect organogenesis of chestnut (*Castanea sativa* Mill.)

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To establish an effective protocol for plant regeneration through indirect organogenesis, effects of explants type, culture media and plant growth regulators on callus induction and shoot regeneration of chestnut (Castanea sativa Mill.) were investigated. Three different explants (root, nodal and internodal segment), two different media [Murashige and Skoog medium (MS) and Gamborg's B5 (B5)] and different plant growth regulators (6-benzylaminopurine (BA), thidiazuron (TDZ), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA)) with different concentration (0.2, 0.5, 1 and 1.5 mgL^{-1}) for shoot and root induction were chosen. The results show that nodal segment was the best explant for callus induction (69.4%) when cultured on MS medium supplemented with 1 mgL^{-1} TDZ and MS was the best medium to induce callus formation (74.6%). The highest shoot multiplication (66.9%) was observed on MS medium with 0.2 mgL^{-1} TDZ. Regenerated shoots were rooted in vitro on MS containing 1.5 mgL^{-1} IBA. Also, plantlets with well developed root and shoot systems were acclimatized inside the green house and 80% of the plantlets survived on transfer to garden soil. This protocol provides a basis for future studies on genetic improvement.

Key words: Chestnut, node, internode segment, indirect organogenesis, callus formation, shoot regeneration.

INTRODUCTION

For a long time chestnut has been an important economic resource in Europe and more recently in Asia, also playing an important environmental role in many agrofo-restry systems (Bounous, 2005). Chestnut is a hardwood forest species of considerable agro-economic important tree species for both timber and nut production. However, this tree species is threatened by pollution, social and economic changes, and two major fungal diseases; ink disease (*Phytophthora* sp.) and chestnut blight [*Cryphonectria parasitica* (Murr.) Barr.] (Sauer and Wilhelm, 2005). Chestnut is a woody species, which is difficult to propagate either generatively by seed or vegetatively by grafting or cuttings (Osterc et al., 2005). However, as an alternative to conventional vegetative propagation methods, efforts have been made to establish reliable *in vitro* regeneration systems that allow clonal propagation (Vietez and Merkle, 2005; Troch et al., 2010). *In vitro* tissue culture techniques have been applied to chestnut regeneration since the 1980's (Rodriguez, 1982; Vieitez et al., 1983). Also *in vitro* establishment in chestnut is possible from both juvenile and mature material (Sánchez et al., 1997) and explants such as cotyledonary node (San-José et al., 2001), bud (Vieitez and Vieitez, 1980), nodal segment (Osterc et al., 2005), etc have been utilized for *in vitro* propagation of chestnut.

Production of regenerated plant through indirect orga-nogenesis is one of the possible ways to contribute to genetic improvement because there are some advantages

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Abbreviations: BA, 6-Benzylaminopurine; **IAA,** indole-3-acetic acid; **TDZ,** thidiazuron; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium; **B_5,** Gamborg's **B_5.**

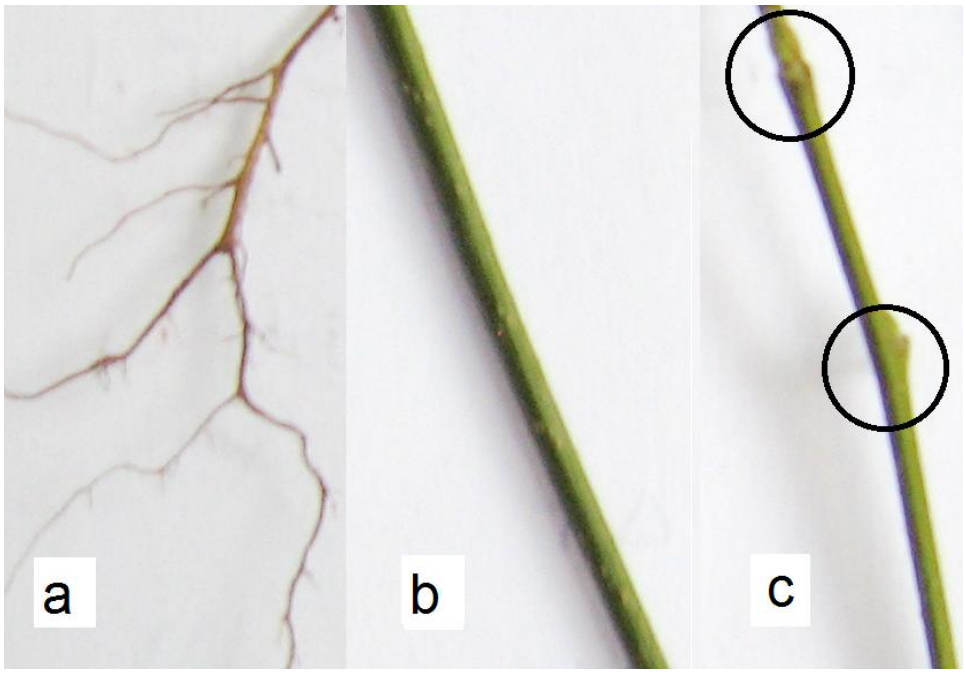

Figure 1. The explants used for experiments (a) root (b) internodal segments and (c) nodal segments.

of shoot regeneration from callus over direct shoot regeneration (Avilés et al., 2009). A callus phase is commonly included in tissue culture protocols with the objectives of generating variability to introduce new desirable traits and generating transgenic plants to introduce traits (Fereol et al., 2005; Zheng et al., 2005). Callogenic initiation implies an initial stage of differentiation from the parental tissue. Thus, to establish callus cultures, the determination of the initial tissue used is a fundamental factor in order to achieve the desired response (Bandyopadhyay et al., 1999). Earlier, zygotic embryos (Kvaalen et al., 2005), hypocotyls (Ahn et al., 2007), ovaries and ovules (Sauer and Wilhelm, 2005), internodes (Chandra and Bhanja, 2002), nodal segments (Arora et al., 2010) and cotyledonary node (Dhabhai and Batra, 2010) have been used in induction of callogenesis and indirect organogenesis.

The aim of this study was to investigate the effects of explant type, culture media and plant growth regulators on callus induction and shoot regeneration from chestnut, and also establish an efficient tissue culture protocol that would provide an efficient tool to be used in chestnut breeding programs.

MATERIALS AND METHODS

Plant materials and culture conditions

Seeds of *Castanea sativa* Mill. were collected from Visroud in Gilan, North of Iran. The area was about 350 ha with 200 to 600 m altitude. The seedlings were grown in a glasshouse under natural illumination (Hou et al*.,* 2010). Root, nodal and internodal segments were removed from three months old seedlings as explants. The excised root, nodal and internodal segments were surface sterilized in 70% ethanol for 1 min followed by 4% sodium hypochlorite for 15 min and rinsed thoroughly three times for 3 min each in sterile distilled water. They were then placed individually (horizontal) in culture vessels (200 × 10 mm) containing 5 ml medium. The Murashige and Skoog, 1962 (MS) and Gamborg et al., 1968 (B_5) media were adjusted to pH 5.8 and 5.5 with 1 N NaOH or HCl, respectively, before autoclaving at 1.06 $kgcm^2$ pressure and 121°C temperature for 20 min. All cultures were incubated at 25 ± 2°C with 16/8 h photoperiod under light from cool white fluorescent lamps (125 µmol $m^{-2}s^{-1}$) and 60 to 70% relative humidity.

Effect of explants type, culture media and plant growth regulators on callus proliferation and shoot regeneration

Root, nodal and internodal segments excised from three-month-old seedlings (Figure 1) were placed on MS medium supplemented with 1 mgL^{-1} thidiazuron (TDZ) to evaluate the effects of different explants on callus proliferation of chestnut as the first experiment. According to the results, nodal segments were the best explants to generate callus. Therefore, the next experiments were done using nodes as initial explants.

For the second experiment, the effects of MS and B_5 basal media supplemented with 1 mgL^{-1} TDZ on callus proliferation from nodal segments were evaluated. According to the results, MS medium was better than B_5 medium to induce callus formation from nodes. Therefore, the next experiments were performed using nodal segments cultured in MS medium.

The objective of the third experiment was to investigate the effects of growth regulators on callus proliferation and shoot regeneration from chestnut. We used MS medium containing different concentration of TDZ and 6-benzylaminopurine (BA) (0.2, 0.5, 1 and 1.5 mgL^{-1}). Data collected included percentage of explants

Table 1. Effects of different explants types on callus proliferation from *Castanea sativa* Mill.

Explant	Explant forming callus (%)
Node	69.4 ± 0.40[a]
Internodal segment	39.1 ± 2.64[b]
Roots	14.2 ± 1.36[c]

Data was collected after eight weeks of culture. Explants were cultured on MS medium supplemented with 1 mgL^{-1} TDZ. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to Student-Newman-Keuls multiple comparison test ($P<0.05$). TDZ, Thidiazuron; MS, Murashige and Skoog medium.

Figure 2. Plant regeneration from callus derived from *in vitro* cultured nodal and internodal segment of *Castanea sativa* Mill. (a) Callus formation from node on MS medium with 1 mgL^{-1} TDZ after four weeks of culture. (b) Callus formation from root segments on MS medium with 1 mgL^{-1} TDZ after four weeks of culture. (c) Callus formation from internodal stem on MS medium with 1 mgL^{-1} TDZ after four weeks of culture. TDZ, Thidiazuron; MS, Murashige and Skoog medium.

forming callus, shoot proliferation percent, mean number and length of shoots after eight weeks of culture. Explants were subcultured onto the MS medium with same BA and TDZ concentration at two weeks for further callus and/or shoot development.

Root induction

When shoots developed from initial explants and became 20 to 30 mm in length about eight weeks after culture, they were excised and transferred to the rooting medium. The rooting medium consisted of MS medium supplemented with different concentration of IBA and indole-3-acetic acid (IAA) (0.2, 0.5, 1 and 1.5 mgL^{-1}). The percentage frequency of root formation and its length was calculated after four weeks of culture. The rooted plantlets (four to five weeks old) were then taken out from the culture vessels, washed thoroughly in running tap water to remove any remains of the nutrient-agar medium. Therefore, they were planted into pots (10 cm in diameter), containing a mixture of sterile vermiculite and sand (1:1) and maintained in the growth chamber at a temperature of 25 to 28°C, 16/8 h photoperiod and relative humidity of 80 to 90% covered with plastic bags. Once established in soil, the plants were transferred to the greenhouse at a temperature of 25 to 28°C and 16/8 h photoperiod.

Statistical analysis

The experimental unit was culture vessels. This treatment had five replications with 10 explants plated for each replication. For the comparison between MS and B_5 media, we used T-test. A completely random design was used for the data analysis. Results were analyzed statistically using the statistical analysis system program (SAS, 2001). The mean values were calculated and compared by Student-Newman-Keuls multiple comparison test ($P<0.05$).

RESULTS AND DISCUSSION

Effect of explants type, culture media and plant growth regulators on callus proliferation and shoot regeneration

In *C. sativa* Mill., callus formation varied significantly depending on explant type (Table 1). Nodal segments showed the earliest signs of callus formation from the cut edges on MS medium supplemented with 1 mgL^{-1} TDZ after one week of culture, but root segments and internodal segments started to initiate callus from cut surfaces after two weeks of culture. Figure 2 indicate the callus of different explants after four weeks. The nodal segments showed higher callus formation, while roots and internodal segments exhibited a significantly lower callus induction. The highest callus induction (74.6%) was achieved when MS medium was used in comparison with 69.1% on B_5 medium (Table 2).

Table 2. Effects of different culture media on callus proliferation from node of *Castanea. sativa* Mill.

Medium	Explant forming callus (%)
MS	74.6 ± 1.67^{a}
B_5	68.1 ± 1.12^{b}

Data was collected after eight weeks of culture. Different media were supplemented with 1 mgL^{-1} TDZ. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to T-test ($P<0.05$). TDZ, Thidiazuron; MS, Murashige and Skoog medium; B_5, Gamborg's B_5.

Table 3. Effects of different growth regulators on callus proliferation and shoot regeneration from nodal segments of chestnut (*Castanea sativa* Mill.)

Growth regulator (mgL^{-1})		Explant forming callus (%)	Explant regeneration shoot (%)	Mean shoot length (cm)
BA	TDZ			
0.2	0	64.3 ± 0.28^{f}	24.7 ± 2.24^{e}	0.5 ± 1.42^{e}
0.5	0	67.6 ± 0.16^{ef}	31.8 ± 2.73^{d}	0.6 ± 1.94^{e}
1	0	72.3 ± 0.43^{c}	32.6 ± 2.0^{e}	0.6 ± 2.13^{e}
1.5	0	76.9 ± 0.67^{b}	51.5 ± 2.91^{b}	1.6 ± 1.25^{c}
0	0.2	85.3 ± 0.68^{a}	66.9 ± 2.13^{a}	2.2 ± 1.44^{a}
0	0.5	71.2 ± 0.22^{d}	53.3 ± 2.66^{b}	1.9 ± 1.67^{b}
0	1	79.4 ± 0.40^{e}	41.4 ± 2.84^{c}	1.4 ± 1.25^{d}
0	1.5	60.8 ± 0.29^{e}	39.1 ± 2.0^{d}	0.9 ± 2.85^{e}

Data was collected after eight weeks of culture. Nodal segments were cultured on MS basal medium. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to Student-Newman-Keuls test ($P<0.05$). BA, 6-benzylaminopurine; TDZ, thidiazuron; MS, Murashige and Skoog medium.

The variations of callus induction on different media may be due to the differences of nitrate/ammonium (NO_3^-/NH_4^+) ratio, an important factor on nitrogen uptake and pH regulation during plant tissue culture (Fracago and Echeverrigaray, 2001). Vieitez et al. (1983) compared several media to obtain the cluster propagation ability in different chestnut clones from the same hybrid group *Castanea sativa* × *Castanea crenata*. The highest number of shoots per culture formed was achieved when the explants were grown on Heller's medium; explants grown on Heller's+$(NH_4)_2SO_4$ medium (Heller, 1953) and on MS medium with the addition of ammonium nitrate (NH_4NO_3) were slightly worse. However, Ballester et al. (2001) indicated that it is very difficult to recommend a mineral medium for general application; nevertheless, half strength MS media appeared to be the most suitable for multiplication through axillary shoot development. Therefore, it is concluded that the best basal medium for the callus induction was MS, although successful callus formation has been achieved on B_5 medium (Table 2). An increase in BA concentration from 0.2 to 1.5 mgL^{-1} increased callus induction from 64.3 to 76.9. The highest frequency of callus formation (85.3%) was obtained on MS medium containing 0.2 mgL^{-1} TDZ.

Shoot regeneration

Calli formed numerous shoots when they were cultured on the same medium after three to four weeks of culture (Table 3). Cytokinin type and concentration also affected the frequency of shoot induction (Maheshwari and Kovalchuk, 2011). In the present experiment, BA could induce shoot regeneration from nodal segments at the percent of 76.9% when cultured on MS medium with 1.5 mgL^{-1} BA. Increasing BA from 0.2 to 1.5 mgL^{-1} resulted in an increase in shoot regeneration ability in callus (Table 3). Vieitez and Vieitez (1980) reported that 6-benzylaminopurine (BAP) showed the most satisfactory effect on promoting the proliferation of axillary shoots, whereas zeatin slightly inhibited the development of axillary shoots but increased the induction rate and caused more vigorous shoots. Tetsumura and Yamashita (2004) achieved similar results with the addition of zeatin also causing the highest proliferation rate. In addition, Arora et al. (2010) found that it is possible to obtain shoot regeneration through indirect organogenesis from nodal segment of *Azadirachta indica* A. Juss. on MS medium supplemented with 0.2 mgL^{-1} BA. Girijashankar (2011) achieved shoot regeneration of *Acacia auriculiformis* from

Table 4. Effect of different concentrations of IBA and IAA on root induction in shoots of chestnut (*Castanea sativa* Mill.)

Growth regulators (mgL^{-1})		Rooting (%)	Mean root length (cm)
IBA	IAA		
0.2	0	51.2 ± 1.54^{d}	0.7 ± 1.43^{e}
0.5	0	52.3 ± 1.94^{d}	1.7 ± 2.25^{c}
1	0	68.3 ± 1.11^{b}	1.6 ± 2.17^{c}
1.5	0	71.2 ± 1.88^{a}	2.1 ± 0.73^{a}
0	0.2	41.5 ± 2.27^{e}	1.0 ± 1.14^{de}
0	0.5	40.2 ± 1.01^{e}	1.4 ± 1.20^{d}
0	1	51.1 ± 1.32^{d}	1.7 ± 0.94^{c}
0	1.5	64.2 ± 1.99^{c}	2.0 ± 1.61^{b}

Data was collected after four weeks of culture. Nodal segments were cultured on MS basal medium. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to Student-Newman-Keuls test ($P<0.05$). IAA, Indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium.

nodal stem segments on MS containing BA (2 mgL^{-1}) + naphthaleneacetic acid (NAA) (1 mgL^{-1}). Also single nodal segments of *Castanea mollissima* cv. 'yanshanhong' induced shoot on MS medium with a half concentration of NO_3 supplemented with 0.5 mgL^{-1} BA (Hou et al*.,* 2010). The result of present study shows that TDZ induced more shoot regeneration. The highest shoot regeneration (66.9%) was obtained on MS medium supplemented with 0.2 mgL^{-1} TDZ, while further addition of TDZ concentration resulted in decreased shoot regeneration. The lowest percentage of shoot induction was observed on MS medium with 1 mgL^{-1} BA.

TDZ has been shown to be the most effective in inducing shoot regeneration in woody species and has shown to have a much stronger ability than BA on shoot induction (Huetteman and Preece, 1993). It is a synthetic polyurea which being a cytokinin-like substance, is highly effective for shoot regeneration in tissue culture of recalcitrant plant species (Liu et al., 2003). However, if concentrations of TDZ are high, hyperhydricity or morphological abnormalities could be observed among regenerated shoots (Huetteman and Preece, 1993). Lower concentrations of TDZ are thus preferable for shoot regeneration (Wang et al., 2008). In the current study, the maximum length of shoots was observed from nodal segments in MS supplemented with 0.2 mgL^{-1} TDZ (2.2 cm) (Table 3). The cytokinins, because of their role in experimentally induced cell division and differentiation, serve as a probe of hormonal involvement in differentiation (Hall, 1976). This study demonstrate the superiority of TDZ over BA as shoot-inducing cytokinin in the *in vitro* induction of adventitious shoots from nodal segments of chestnut. Ballester et al. (2001) also used 0/1 mgL^{-1} TDZ in MS medium to germinate chestnut embryonic axes.

Root induction from shoots

Rooting of the regenerated shoots did not occur on the shoot induction medium. Elongated shoots (20 to 30 mm in length) regenerated on MS medium were used for rooting experiment. Auxins had a significant influence on root formation. Depending on auxin type and concentration, roots were initiated between five to 15 days of culture. The best root formation and maximum mean length (2.1 cm) was observed during regeneration of chestnut on MS basal medium supplemented with 1.5 mgL^{-1} IBA (the root regeneration percent was 71.2%) (Table 4). IAA at 1.5 mgL^{-1} also induced 64.2% rooting in regenerated shoots, but the number of roots per shoot was considerably lower. The roots were regenerated within two weeks after transferring onto these media. The roots regenerated on the medium supplemented with 1.5 mgL^{-1} IBA were longer than others (Table 4).

Among the auxins, IAA and IBA are the most frequently applied chemicals for rooting (Harry and Thrope, 1994). Our results also show that IBA was the best one to be used for root formation. Similar results about response to IBA are observed in *Prosopis ceneria* for root induction (Kumar and Singh, 2009) and also *Azadirachta indica* A. Juss (Chaturvedi et al., 2004). Figure 3 shows the whole plantlet from nodal segment on MS with 0.2 mgL^{-1} TDZ and then 1.5 mgL^{-1} IBA. *In vitro* raised plantlets with well developed shoots and roots were transferred to pots containing sterile soilrite and acclimated, after which the successfully acclimated plants (80%) were transferred to pots under full sun where they grew well without any detectable phenotypic variation. Ahn et al. (2007) indicated that for plant regeneration from hypocotyls of *Ricinus communis* L., TDZ induced adventitious shoots at a higher rate compared to shoots induced by BA and also IBA was more efficient in root growth and shoot development than NAA.

Conclusion

The limitation of this study lies on the experimental materials, which were from two to three months old

Figure 3 Plantlet from nodal segment via callus on MS with 0.2 mgl^{-1} TDZ and then 1.5 mgl^{-1} IBA.

seedlings instead of mature trees with good characters. Seedling materials cannot completely represent the mature ones because of the difference of explants condition (Hou et al., 2010). However, for obtaining the physiology mechanism of adventitious root formation of *C. sativa Mill.* in a comparative short time, this experiment should be the guide of further research. We were successful in plant regeneration in *C. sativa* Mill. using MS medium supplemented with 0.2 mgL^{-1} concentration of TDZ and 1.5 mgL^{-1} IBA. This regeneration protocol will be useful not only for further research studies such as genetic cell transformation or protoplast fusion studies, but also for commercial nurseries that could use virus-free plants and agricultural practices to reduce pesticide use and increase yield production.

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