

Full Length Research Paper

Callus production and regeneration of the medicinal plant *Papaver orientale*

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***Papaver orientale* is an important medicinal plant and source of the valuable alkaloids. In this study, callus production and regeneration of *P. orientale* under *in vitro* conditions was investigated with different levels of kinetin (Kin), 6-benzylamine purine (BA) and α -naphthalene acetic acid (NAA) on Murashige and Skoog (MS) and Gamborg's B5 (B5) media. For callogenesis, explants as cotyledons-hypocotyls were placed on the solidified MS and B5 media with hormones under 16-h photoperiod and 20°C conditions. Results showed that the best media for callus induction of *P. orientale* consisted of the MS basic media supplemented with 0.5 mg/l BA and 0.5 or 1 mg/l NAA. Shoots were regenerated in cultures grown on MS medium containing 0.5 mg/l Kin and (0.5 or 1.0 mg/l) NAA and were induced to root on B5 medium containing 1 mg/l Kin and 1.0 mg/l NAA.**

Key word: Callus induction, *in vitro* regeneration, Papaveraceae, *Papaver orientale*.

INTRODUCTION

Papaveraceae family have many applications including inhibition of acetylcholinesterase (Cahlikova et al., 2010), antibacterial agent (Bhattacharjee et al., 2010) or inhibition of algae and cyanobacteria in water management (Jancula et al., 2007, 2010). The genus *Papaver* L. is of commercial interest because of its medicinally important alkaloids. *Papaver* species produce a wide range of isoquinolines, sometimes with very high yields (Phillipson, 1983). Isolation of medicinally important alkaloids, morphine, codeine, papaverine and narcotine has been of considerable interest in the alkaloids of *Papaver* species.

Species of the genus *Papaver* are grouped together based on their morphological characteristics into ten sections namely, Argemonidium, Carinata, Glauca, Horrida, Meconella, Milnthana, Oxytona, Papaver, Pilosa and Rhoeadium. Revision of section Oxytona in *Papaver* by Goldblatt (1974) resulted in the recognition of three species. It was reported that each species could be

characterized morphologically (example, by petal coloring and markings and by capsule shape). Furthermore, the three species were distinguished by cytological and chemical techniques: *Papaver bracteatum*, $2n = 2x = 14$, major alkaloid thebaine; *Papaver orientale*, $2n = 4x = 28$, major alkaloid oripavine and *Papaver pseudo-orientale*, $2n = 6x = 42$, major alkaloid isothebaine. *P. bracteatum* and *P. orientale* are characterized by the high content of their morphinane alkaloids thebaine and oripavine, respectively (Theuns et al., 1987). *P. pseudo-orientals* has isothebaine as a dominant alkaloid, and morphinane alkaloids may be found in trace amounts (Böhm and Nixdorf, 1983). Dry latex of *P. orientale* has been shown to contain 20% oripavine and 9% thebaine (Shafiee et al., 1975).

Media after Murashige and Skoog (1962) and Gamborg et al. (1968) are most commonly used for *Papaver* tissue cultures and have supported successful alkaloid production. Tissue cultures of *Papaver somniferum* have been grown in modified MS media and B5 medium. *Papaver* callus grown *in vitro* produced morphinane alkaloids in low concentrations. Yoshikawa and Furuya (1985) gave an account of the production of codeine and thebaine in a green callus with relatively high levels of kinetin.

Schuchmann and Wellmann (1983) reported that high yield embryogenesis could be induced by tissue cultures

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Abbreviations: BA, 6-Benzylamine purine; 2,4-D, 2,4-dichlorophenoxyacetic acid; DMR, Duncan's multiple range; MS, Murashige and Skoog; B5, Gamborg's B5 medium; Kin, kinetin; NAA, α -naphthalene acetic acid.

Table 1. Callus, shoot and root induction rate (%), callus, shoot and root fresh weight (mg) of cotyledon-hypocotyl of *P. orientale* with various combinations and concentrations of cytokinins and auxins.

medium	Supplemented with (mg/l)			Callus induction percent	Callus fresh weight (mg)	Shoot induction percent	Shoot fresh weight (mg)	Root induction percent	Root fresh weight (mg)
	NAA	BA	Kin						
MS	0.5	0.5	0	97.7 ^{A*}	1857.8 ^A	17 ^E	1154.4 ^{BC}	0	0
	1	0.5	0	100 ^A	1756.6 ^A	32 ^D	1367 ^B	0	0
	0.5	1	0	83.9 ^B	1394.6 ^B	46 ^C	1002 ^C	0	0
	1	1	0	72.2 ^C	733.4 ^{DE}	19.1 ^E	1083.2 ^C	0	0
	0.5	0	0.5	51.2 ^{DE}	886.4 ^D	71.4 ^A	1399.4 ^B	21.7 ^C	25 ^B
	1	0	0.5	71.9 ^C	1331.6 ^B	66.7 ^{AB}	1738.4 ^A	23.3 ^C	18 ^C
	0.5	0	1	0	0	0	0	0	0
	1	0	1	18.3 ^G	443.6 ^F	0	0	0	0
B5	0.5	0.5	0	46.8 ^{EF}	1097.6 ^C	0	0	23.4 ^C	18.6 ^C
	1	0.5	0	49.6 ^E	647 ^E	0	0	0	0
	0.5	1	0	0	0	0	0	0	0
	1	1	0	39.3 ^F	738.4 ^E	0	0	0	0
	0.5	0	0.5	12.5 ^G	675.4 ^E	17.2 ^E	1025.8 ^C	46.7 ^{AB}	28 ^B
	1	0	0.5	60.3 ^D	1087.4 ^C	0	0	35.4 ^{BC}	19.4 ^C
	0.5	0	1	17.5 ^G	774.8 ^{DE}	58.9 ^B	1029.4 ^C	40.2 ^B	28 ^B
	1	0	1	37.8 ^F	0	0	0	58.9 ^A	33 ^A

*Superscript letters show treatment means, which were separated by Duncan multiple range test (DMRT) at 5% level. Treatments with common superscripts do not have significant differences at $p < 0.05$.

of *P. somniferum* or *P. orientale* in 2,4-dichlorophenoxyacetic acid (2,4-D) free Gamborg's B5-medium. Daneshvar (2005) reported that the best shoot regeneration of *P. pseudo-orientale* was obtained from hypocotyl-cotyledon explant on MS medium containing 0.24 mg/l 2,4-D and 0.19 mg/l α -naphthalene acetic acid (NAA). The rooting was obtained on MS medium containing 0.25, 0.50 and 1.00 mg/l IBA with the best results on MS medium containing 0.5 mg/l IBA. Rostampour et al. (2010) reported that the optimized callus induction media for *P. bracteatum* consisted of the MS media supplemented with 1.0 mg/l 2,4-D, 0.1 mg/l kinetin (Kin) and 15 mg/l ascorbic acid.

Kassem and Jacquin (2001) reported that the hypocotyls and cotyledons both show somatic embryogenesis in *P. somniferum album*, whereas only the cotyledons were embryogenic in *P. orientale*. Numerous studies have shown that the production of morphinan alkaloids via *in vitro* cultures requires organogenesis of tissues in cultures (Schuchmann and Wellmann, 1983; Kamo et al., 1982).

We studied the influence of different concentrations of plant growth regulators and different culture media on callus production and regeneration in *P. orientale*. The findings of this study would help in the production of alkaloids of this species under controlled conditions.

MATERIALS AND METHODS

Plant material and seed germination

Seeds of *P. orientale* were collected from Ardabil region E 48° 49' 39" and N 38° 50' 22", northwest of Iran. Seeds were surface-sterilized using 70% (v/v) ethanol for 1 min followed by three-times washing with distilled sterile water. They were then kept in 1% (v/v) sodium hypochlorite solution for 30 min followed by three washes with distilled sterile water and germinated on MS under 16/8 h (light/dark) photoperiod at 20 °C temperature. The cotyledons and hypocotyls were cut into 5 ± 1 mm segments from 10 day-old seedlings, and used as explants.

Media and culture conditions

The basal medium consisted of B5 or MS salts with 3% sucrose (w/v) and solidified with 7% (w/v) agar. The media were adjusted to pH 5.7 with 1.0 M NaOH or HCl before adding agar, and then sterilized by autoclaving at 121 °C for 20 min.

Callus induction

The explants from cotyledons-hypocotyls were placed on B5 (Gamborg et al., 1968) and MS (Murashige and Skoog, 1962) media supplemented with kinetin or benzylaminopurine (BA), and NAA at different combinations (Table 1). All media were supplemented with 3% sucrose, and 15.0 mg l⁻¹ ascorbic acid. For callus induction, cotyledons-hypocotyls explants were placed on 25

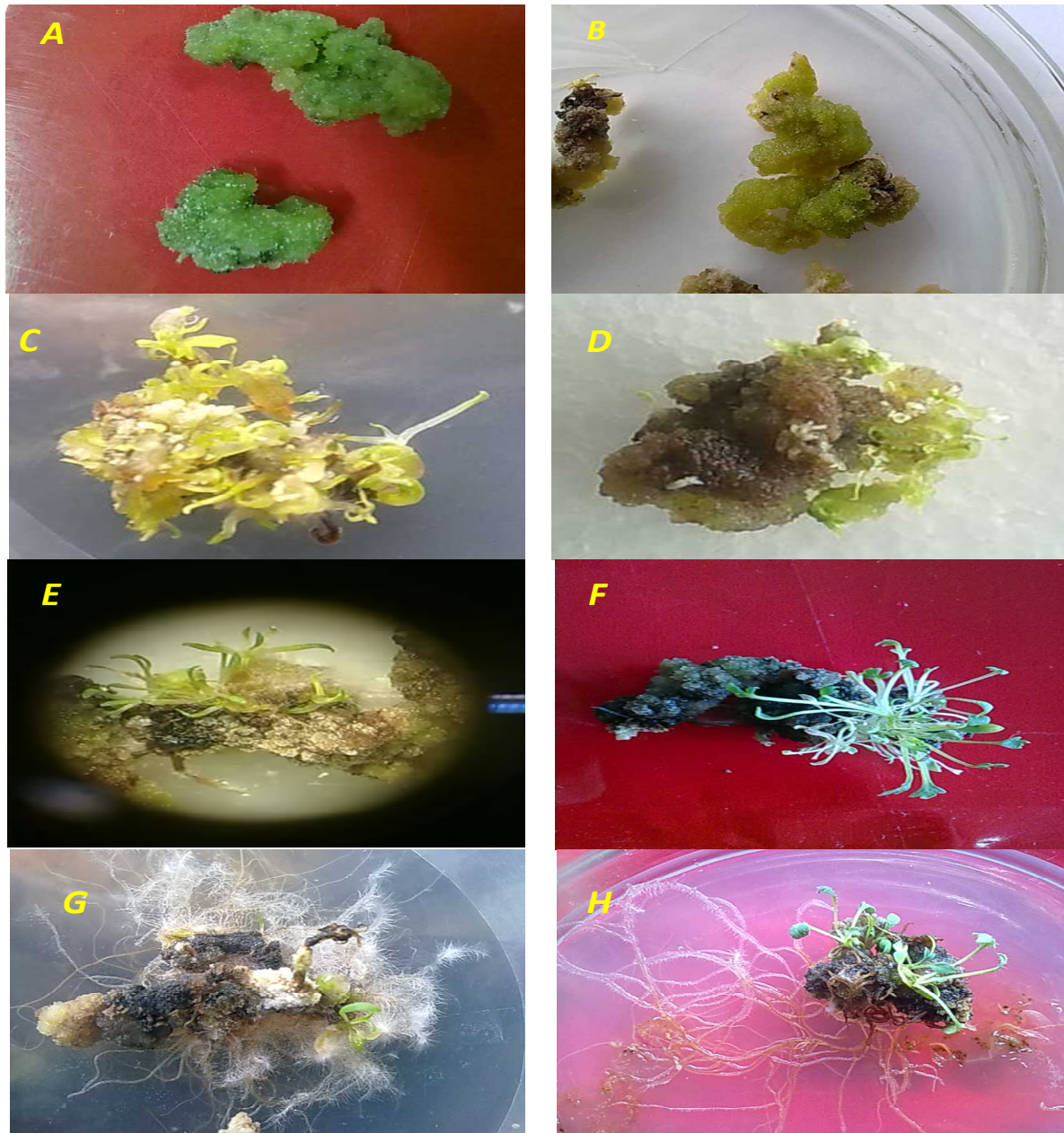


Figure 1. Callus induction and plant regeneration of *P. orientale*. A and B: Green callus from explants after two months of culture; C, D and E: shoots regeneration from callus three months of culture; F: shoots regeneration from callus after four months of culture; G and H: roots regeneration from callus.

ml of agar-solidified medium in Petri dishes, sealed with parafilm and incubated at 20°C under 16 h light/8 h dark photoperiod. Well-grown callus induced from explants were transferred to the original media and sub-cultured every 20 days.

Plant regeneration

Preliminary experiments for testing the potential of regeneration of calli were performed using different combinations and concentrations of the earlier-mentioned cytokinins and auxins. Based on the test, calli were transferred into the MS medium containing

various combinations and concentrations (0.5 and 1.0 mg/l) of Kin, BA and NAA for regeneration. All the cultures were then incubated at 20°C under 16 h light/8 h dark photoperiod.

Statistical analysis

In this study, percentage of callus induction, callus fresh weight (mg), shoot and root induction percent and shoot and root fresh weights (mg) were measured in each treatments. Test of significance was carried out by ANOVA and the data means were compared by Duncan multiple range tests (DMRT) using the SPSS

software.

RESULTS AND DISCUSSION

In this study, two different media for plant tissue culture (MS and B5) and various combinations or concentrations of growth regulators were used to find out their effects on callus induction and plant regeneration. As shown in Figure 1, callus formation started from cotyledonhypocotyls explants after one week of culturing on the callus induction media. In preliminary experiments, the calli showed browning when the culture period was extended further with 4 weeks due to prolonged period of culture on the same medium and the production of phenolic compounds. Adding the ascorbic acid (15 mg/l) to the basal medium was effective to control browning.

Results showed that MS medium was more effective than B5 medium for callus induction in *P. orientale* (Table 1). Among the cytokinins, the BA was more effective than Kin for callus induction when used in combination with NAA (Table 1). This study showed that the best medium for callus induction in *P. orientale* was MS supplemented with 0.5 mg/l BA and 0.5 or 1.0 mg/l NAA with an average of 97.7 and 100% explants producing callus (Table 1). Increasing BA concentration from 0.5 to 1 mg/l caused a reduction of callus production and this reduction was greater at 1 mg/l concentration of NAA. Other hormone combinations, such as 0.5 mg/l NAA with 1 mg/l BA in B5 medium and 0.5 mg/l NAA with 1 mg/l Kin in MS medium were ineffective for callus inducing in *P. orientale* (Table 1). Day et al. (1986) have also reported callus and meristemoid formation in *P. bracteatum* Arya II cultures on MS containing 2,4-D and Kin. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and usually used together (Gang et al. 2003). It was revealed that auxins played an important role in the callus induction and different types of auxins had various effects (Skoog and Armstrong 1970; Baskaran et al., 2006), and the cytokinins facilitated the effect of auxin in callus induction (Rao et al., 2006; Yang et al., 2008). Ilahi and Ghauri (1994) reported that callus was induced in *P. bracteatum* Lindl. seedlings inoculated on MS medium supplemented with NAA (1.0 mg/l) and BA (0.5 mg/l).

Shoot formation started in callus after 8 weeks of culturing on the shoot induction media (Figure 1). This study showed that the most favorable medium for shoot induction was MS supplemented with 0.5 mg/l Kin and 0.5 mg/l NAA with an average of 71.4% shoot induction rate (Table 1). Other growth-regulator combinations, failed to induce shoots in *P. orientale* (Table 1). The results showed that MS medium was more effective than B5 medium and Kin was more effective than BA, which is crucial for high shoot induction of *P. orientale*. Ilahi and Ghauri (1994) reported that shoots were regenerated in cultures grown on MS medium containing NAA (1.0 mg/l), BA (0.5 mg/l) and casein hydrolyzate (2.0 mg/l).

Root formation started in callus after 16 weeks of subculturing on the root induction media (Figure 1). This study showed the optimal medium for root induction was B5 medium supplemented with 1.0 mg/l Kin and 1.0 mg/l NAA with an average of 58.9% (Table 1). Other growth-regulator combinations, failed to induce roots from calli of *P. orientale*. These results showed that B5 medium was more effective than MS medium and Kin was more effective than BA, which is crucial for high frequency root induction of *P. orientale* (Table 1).

In conclusion, we reported an efficient *in vitro* regeneration system of *P. orientale* and the results could be helpful not only for large scale vegetative propagation, but also for genetic improvement of *P. orientale* through transformation studies. Optimized callus induction media for *P. orientale* consisted of the MS supplemented with 0.5 mg/l BA and 0.5 or 1.0 mg/l NAA. In this species, shoots were regenerated in cultures grown on MS medium containing 0.5 mg/l Kin and 0.5 or 1.0 mg/l NAA and were induced to root on B5 medium containing 0.5 mg/l Kin and NAA or 1.0 mg/l Kin and NAA.

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