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Full Length Research Paper

Establishment of *in vitro* plant regeneration system for *Chimonanthus praecox* (L.)

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An efficient protocol for plant regeneration of *Chimonanthus praecox* (L.) Link, was developed using leaves from seedlings of seeds. Shoots induction were influenced by cytokinins and nodal positions. The results show that the highest callus induction frequency was obtained on MS medium supplemented with 2.0 mg l^{-1} 6-benzyladenine (6-BA) and 0.9 mg l^{-1} 1-naphthalene-acetic acid (NAA) after 10 days incubation in darkness. The frequency of callus induction of different nodal leaves (from the first to the forth node) varied from 70.0 to 93.5%; the highest frequency of callus induction (93.5%) was observed in the second node group. Through many times subculture of callus, green and compact callus was selected and transferred to regeneration medium (MS, 1/2 MS and woody plant medium (WPM) supplemented with different concentrations of thidiazuron (TDZ) (2.37, 4.74 and 9.48 mg l^{-1}) and NAA (0.74, 1.48, and 2.96 mg l^{-1}). It was shown that the optimal combination of the three factors was WPM + NAA + TDZ. The highest adventitious shoot regeneration frequency of leaf explants (71.33%) and the highest mean number of shoots per explant (2.23) were obtained on WPM supplemented with 2.37 mg l^{-1} TDZ and 1.48 mg l^{-1} NAA. Optimal rooting was seen on 1/2 MS medium with 1.5 mg l^{-1} IBA for eight days, followed by transfer to hormone-free MS medium. Rooted plantlets were easily acclimated in a glasshouse.

Key words: *Chimonanthus praecox*, *in vitro* regeneration, adventitious buds.

INTRODUCTION

Calycanthus chinensis (L.) is the only representative species in the genus in China. It is endemic to China. In Europe, it is grown mostly in parks and urban green areas, but in China, it is a widespread floricultural species and is a 2.5 to 3.0 m tall deciduous shrub. *C. praecox* is a rare ornamental species that flowers during winter in the Northern Hemisphere and the bright and scented flowers appear from November to February, usually in mid-January. It is frost hardy, surviving short periods of frosts with temperatures reaching as low as -18°C. Known active compounds present in its tissues include chimonamidine, triptamine type alkaloids which have important biological activities (Takayama et al., 2004; Kitajima et al., 2006). So, the species is well-known in Chinese folk medicine where it is used for the treatment of rheumatic arthritis (Takayama et al., 2004).

Vegetative propagation of *C. praecox* is usually done by spring cuttings or layering. However, only one recent report has dealt with micropropagation or *in vitro* culture of *C. praecox* (Kozomara et al., 2008), secondly, callus induction and plant regeneration from other plants has been reported by a number of researchers (Al-Khayri et al., 1989; Asano, 1989, Asano et al; 1996; Inokuma et al.,1996; Chai et al., 1998; Veltcheva et al., 2005; Feng et al., 2010), but for callus induction and plant regeneration, there is no data available in the literature. Since *C. praecox* contains a number of interesting secondary metabolites (Iwashina et al., 2001; Takayama et al., 2004; Kitajima et al., 2006), here, we reported an efficient protocol for callus induction and *in vitro* plant

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Abbreviations: KT, Kinetin; **TDZ,** thidiazuron; **ZiP,** N^6-(2-isopenteny) adenine; **MS,** Murashige and Skoog; **NAA,** 1-naphthalene-acetic acid; **IBA,** indole-3-butyric acid; **WPM,** woody plant medium; **PGRs,** plant growth regulators; **6-BA,** 6-benzyladenine.

regeneration of *C. praecox*.

MATERIALS AND METHODS

The materials used here were leaves collected from the top of the second node from seedlings germinated in the glasshouse.

Callus induction

Expanded leaves in the second node of 4-week-old shoots of *C. praecox* were excised and transversely to the midrib, cut into 1 x 1cm size. Then, the leaves were submerged in 70% ethanol for 30 s, washed twice with sterile distilled water, and then immersed in 0.1% $HgCl_2$ solution for 5 to 6 min. The surface-sterilized leaves were then washed three times with sterile distilled water, dried and placed with the abaxial surface contacting MS medium, containing 1.5 g l^{-1} PVP (Sigma-Aldrich, St. Louis, MO USA.) and 20 g l^{-1} sucrose, supplemented with 0.9 mg l^{-1} NAA (Sigma–Aldrich, St. Louis, MO USA.). The medium was adjusted to pH 5.8 after addition of the combination of growth regulators: (6-BA) (Sigma–Aldrich, St. Louis, MO USA), kinetin (KT) (Sigma–Aldrich, St. Louis, MO USA), thidiazuron (TDZ) (Sigma–Aldrich, St. Louis, MO USA) and N^6-(2-isopenteny) adenine (ZiP) (Sigma–Aldrich, St. Louis, MO USA) (2.0 mg l^{-1}), then autoclaving at 121°C for 20 min. After cooling (about 50°C), the medium was poured into 100 ml Erlenmeyer flasks (approximately 40 ml per flask). The explants were cultured in the dark at 25 ± 2°C. Non-contaminated explants were transferred to fresh medium until callus induction.

Regeneration and rooting

After two months, green compact callus was selected and transferred to regeneration medium. The experiment was composed of three factors, and each factor was divided into three levels. These parameters included different basal medium (MS, 1/2 MS and WPM) supplemented with different concentrations of 2.0 mg l^{-1} TDZ and 0.9 mg l^{-1} NAA. Based on the results of orthogonal design experiment, the following one factor experiment was carried out separately. Rooting was performed in two steps using shoots 15 mm or more in length. These were cultured on 1/2 MS media supplemented with 1.5 mg l^{-1} IBA for 2, 5, 8 or 11 days and then transferred to 50 ml of hormone-free agar-solidified 1/2 MS medium containing 1.5 mg l^{-1} PVP and layered with 10 ml of hormone-free 1/2 MS liquid medium. Rooting was achieved in a culture room with a 16-h photoperiod (40 µmol $m^{-2}s^{-1}$ cool white fluorescent irradiance) for four weeks at 25 ± 2°C.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using SAS v. 9.1 (SAS Institute, Cary, NC), and means were compared using Tukey's HDS test ($P \leq 0.05$).

RESULTS AND DISCUSSION

C. praecox is very recalcitrant in tissue culture and little information is available on its regeneration. In the only report on shoot regeneration in *C. praecox* (Kozomara et al., 2008), the authors described the course of shoot regeneration, but did not describe adventitious buds induction from callus, so here, we report an efficient protocol for adventitious buds induction from callus for *C. praecox*. In the experiment, we found that the combination of NAA with lower concentration of cytokinins in the callus induction medium for leaves cultures resulted in the formation of a very compact and light-green callus, which had very high regeneration rates. About five days after inoculation, some leaves were surface-contaminated with fungi and bacteria. Non-contaminated leaves were transferred to fresh medium until free of contamination (about two weeks). In order to decrease contamination from *in vitro* cultures, leaves explants were treated with various combinations of ethanol, $HgCl_2$ and treatment times (data not shown) had limited success. Usually, April to June was the best period for callus induction because of relatively lower rates of contamination. This is possibly because, during this period, the temperature and humidity are low, and the growth of leaves is slower. Usually initiating after two months of subculture, the callus obtained were green, fast-growing, compact and nodular in structure (Figure 1A).

Callus induction was significantly affected by the position of the leaf in the plant and by the cytokinin. Callus induction frequencies ranged from 70.0 to 93.5% in different media. The highest frequency was obtained with MS medium supplemented with 0.9 mg l^{-1} NAA, 2.0 mg l^{-1} KT or 6-BA, for both callus induction when compared with 2.0 mg l^{-1} TDZ or ZiP (Table 1). Callus induction from different nodal leaves (from the first to the forth node) varied from 22.5 to 93.5%. The highest frequencies of callus induction (93.5 %) were observed in the second node (Table 2). The results reveal that young nodal leaves derived from the second node were the ideal explants for callus induction, with MS medium supplemented with 0.9 mg l^{-1} NAA and 2.0 mg l^{-1} KT or 6-BA as the optimal concentration for callus induction.

The formation of highly regenerative callus is usually a long process for *C. praecox*, especially callus induction from its leaves. Long-term maintenance and regeneration capacity of callus is very important for *in vitro* selection and genetic transformation of *C. praecox*. Here, we established an efficient regeneration system. Through many subcultures, the green and compact callus was selected and transferred to regeneration medium. Shoot formation was seen in callus clusters four weeks after transfer to regeneration medium (Figure 1B). The experiment was composed of three factors, and each factor was divided into three levels. These parameters included different basal medium (MS, 1/2 MS and WPM) supplemented with different concentrations of TDZ (2.37, 4.74 and 9.48 mg l^{-1}) and NAA (0.74, 1.48 and 2.96 mg l^{-1}) (Table 3). Based on the results of orthogonal design experiment, the following one factor experiments were carried out separately.

The results of orthogonal experiments are shown in Table 3. From this table, it is clearly seen that components in basal medium and their combination with

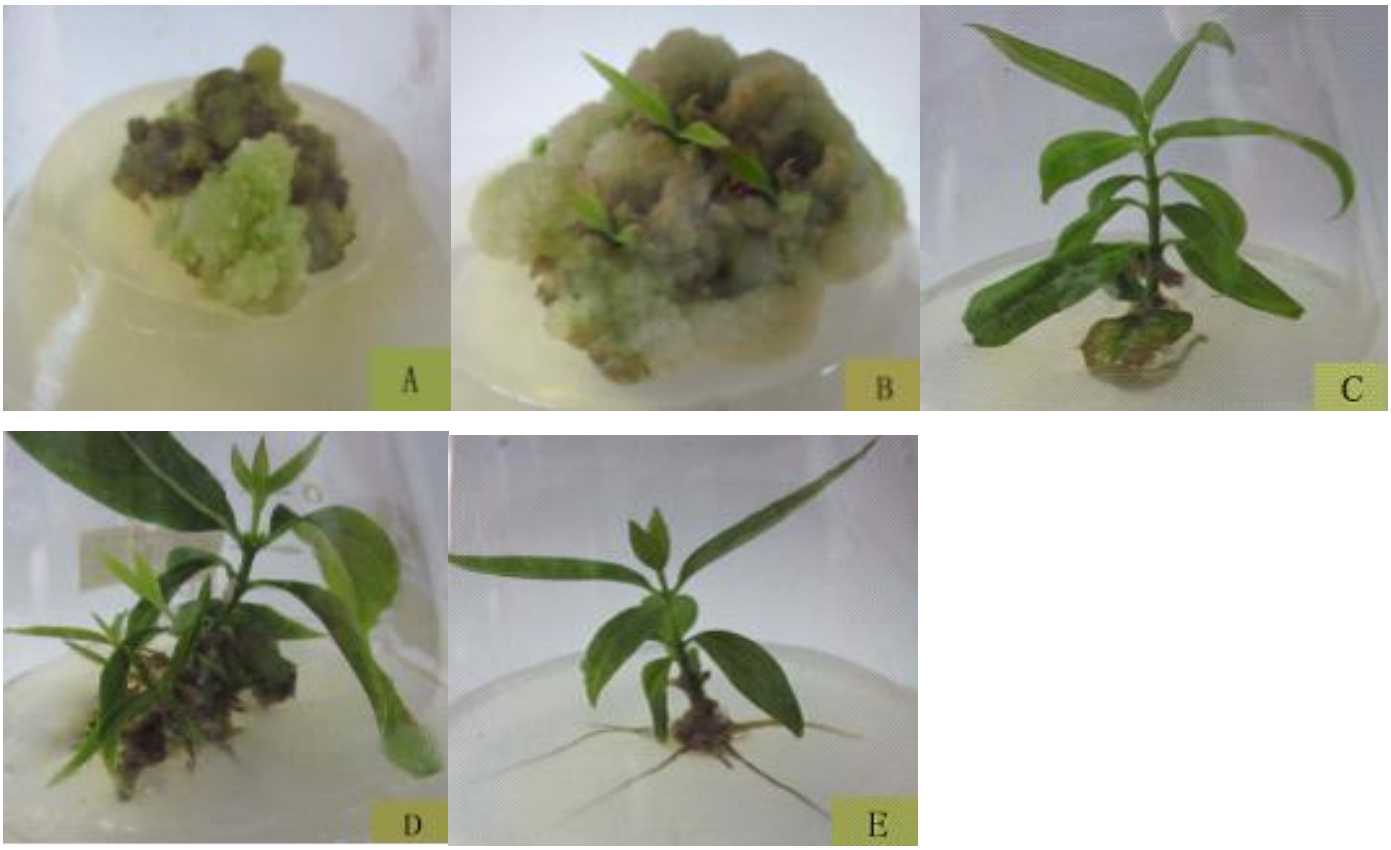

Figure 1. Callus induction, plant regeneration and rooting of *C. praecox*. A, Callus showing green and compact structure. B, Shoot formation from callus clusters four weeks after transfer to regeneration medium. C, Characteristic formation of very large, dark green, leathery leaves and moderate callusing at the shoot base after transfer to regeneration medium. D, Well-established plant clusters 50 days after transfer to the regeneration medium. E, Plant root eight days after transfer to the rooting medium.

Table 1. Effects of medium supplements on callus induction (%) from the second nodal leaves of *C. praecox.*

Plant growth regulator (2.0 mg l^{-1})	Induced callus (%)
6-BA	93.5[a]
KT	91.6[a]
TDZ	70.0[b]
ZiP	88.2[b]

Means indicated with the same letter are not significantly different (HSD Tukey test: $P < 0.05$).

Table 2. Effect of nodal positions on callus induction (%) from the nodal leaves of *C. praecox.*

Nodal position	Induced callus (%)
1	46.7[c]
2	91.6[a]
3	93.5[a]
4	75.4[b]

indicated with the same letter are not significantly different (HSD Tukey test: $P < 0.05$).

The medium was 0.9 mg NAA + 2.0 mg l^{-1} 6-BA. Means be induced after 30 days. It was shown that the optimal combination of the three factors was WPM + NAA + TDZ. The highest adventitious shoots regeneration frequency of leaf explants (71.33%), and the highest mean number of shoots per explant (2.23) were obtained on WPM supplemented with 2.37 mg l^{-1} TDZ and 1.48 mg l^{-1} NAA (Table 3).

After 50 days, the adventitious shoots reached about 30 to 40 mm, they were large, dark green with leathery leaves, and moderate callusing was observed at the shoot base. There were a large number of adventitious shoots that slowly grew from the shoot base (Figure 1D).

Root initiation

For successful rooting, *C. praecox* shoots were cultured for 2 to 11 days on media containing auxins ½ MS-based medium (Table 4). The highest response was obtained when shoots were cultured for eight days on ½ MS medium supplemented with 1.5 mg l^{-1} IBA and then transferred to hormone-free ½ MS medium. This

Table 3. Effect of different basal medium and PGRs concentration on frequency of shoot regeneration (%) and mean number of shoots per explant of *C. praecox.*

S/N	Basal medium	NAA (mg l^{-1})	TDZ (mg l^{-1})	Frequency of shoot regeneration (%)	Mean number of shoots per explant
1	MS	0.74	2.37	61.33 ± 4.70^{b}	1.86
2	MS	1.48	4.74	55 ± 7.06^{c}	1.81
3	MS	2.96	9.48	3.33 ± 00^{f}	1.41
4	1/2 MS	0.74	4.74	11.67 ± 2.37^{e}	1.85
5	1/2 MS	1.48	9.48	8.33 ± 2.37e^{f}	1.47
6	1/2 MS	2.96	2.37	23.33 ± 4.70^{d}	2.05
7	WPM	0.74	9.48	6.67 ± 4.70e^{f}	1.56
8	WPM	1.48	2.37	71.33 ± 4.70^{a}	2.23
9	WPM	2.96	4.74	63 ± 7.06^{b}	1.61

Data recorded after 40-day culture initiation. Mean separation was determined using Duncan's multiple range test, and means with different letters are significantly different at $P < 0.05$.

Table 4. Effect of IBA pretreatment on rooting of regenerated *C. praecox* shoots.

1.5 mg l^{-1} IBA pretreatment (days)	Rooting (%)	Number of root (plant^{-1})	Length of the longest root (mm)
2	22.6	2.0 ± 0.5^{a}	17.2 ± 4.9^{a}
5	67.6	3.0 ± 0.4^{a}	26.6 ± 3.7^{ab}
8	80.0	5.2 ± 0.5^{a}	47.1 ± 2.9^{b}
11	52.2	2.0 ± 0.5^{a}	23.5 ± 9.8^{ab}

Regenerated shoots were given a pretreatment on MS-based media supplemented with IBA after 2 to 11 days. Values in columns are calculated from 60 explants replicated in two independent experiments. Means followed by the same letter are not significantly different at $P \leq 0.05$.

treatment provided the highest rooting percentage (80.0%), the highest number of roots per rooted culture (5.2) and the longest roots (47.1 mm). Successful acclimation of these plantlets was 100%, indicating that these propagated plantlets were fully competent for growth in *in vitro* conditions (Figure 1E).

In conclusion, we established a tissue culture system which can induce very high regeneration rates of embryogenic callus from the leaves of *C. praecox*. The protocol described is efficient and can be used as a means of propagation and multiplication. This protocol has been successfully used in somaclonal variation selection and genetic transformation in our laboratory.

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