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Effects of Standard Basal Salt Mixtures and 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) Urea Pre-treatments on Shoot Regeneration from Mature Cotyledons of Sweet Cherry *in vitro*

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The influences of different basal salt compositions (QL, WPM and B5) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ) pre-treatments on shoot regeneration from sweet cherry cotyledons were investigated. Basal salt composition was critical for obtaining higher regeneration efficiencies from stored cherry cotyledons. TDZ pre-treatments did not enhance shoot regeneration. The highest regeneration efficiencies were obtained with WPM, followed by QL medium. The regeneration ability significantly decreased if B5 basal salt composition was used. WPM significantly increased both regeneration frequencies and shoot number per regenerating explant. In addition, shoot primordia formation on WPM was observable 4 days earlier than B5 medium.

Key Words: Medium salts, 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea, Adventitious shoot, Cherry.

INTRODUCTION

The improvement of sweet cherry (*Prunus avium* L.) *via* conventional breeding methods is a time consuming, difficult and expensive process due to long breeding cycles and lengthy field trial procedures. Alternatively, desired trait could be intoruduced into favourable fruit cultivars *via* genetic transformation[1].

Availability of an efficient and reliable regeneration protocol is the first pre-requisite to develop a transformation technology for a species. Regeneration protocols were developed for most of the *Prunus* species including almond[2], peach[3], plum[4], apricot[5] and sour cherry[6] and genetic transformation is a routine for most *Prunus* species such as peach[7] (*P. persica*), almond[8] (*P. dulcis* Mill.), plum[9] (*Prunus domestica* L.), sour cherry[10] (*P. cerasus* L.) and apricot[11] (*P. armeniaca*).

Although there have been several reports on regeneration of sweet cherry[12-14], it is still among the species that has not been transformed yet. This necessitates conduction of further regeneration experiments for sweet cherry. Several factors are known to effect regeneration such as plant growth regulators, genotype, media, pretreatments, light regime and explant type. Among these media[12,15] and plant growth[6] regulators have recently been successfully used to optimize regeneration by several researchers in different plant genotypes. In this study, effects of different standard salt mixtures (QL[16], WPM[17] and B5[18]) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ) pretreatments were evaluated to optimize adventitious shoot regeneration in mature sweet cherry cotyledons.

EXPERIMENTAL

Explant preparation: Mature fruits of cherry were harvested from cv. 'vista', 'Heid' and 'Tehranivce'. The fruit flesh was removed. Then, the seeds were washed with regular tap water and disinfected with a 0.05 % sodium hypochlorite solution for 15 min. After rinsing with water 4 times, the seeds were dried at room temperature for 3 d on a bench and stored at 4 ºC. At the time of experiment, endocarps were cracked with a hammer to obtain the seeds and the seeds were sterilized again for 15 min using 0.525 % sodium hypochlorite. Then, the seeds were rinsed three times with sterile distilled water and imbibed overnight in final rinsing water. After the removal of the seed coat, the embryonic axis was separated from the cotyledons by removing small slices from both sides of cotyledons where the embryonic axis was attached. Each replicate contained ten cotyledon explants which are placed on shoot induction medium adaxial sides up and each treatment was replicated three times.

Shoot regeneration medium and culture room conditions: Shoot regeneration experiments were carried on media consisted of one of the following three different standard basal salt mixtures as different treatments; QL[16], WPM[17] or B5[18], each supplemented with 25 g L^{-1} sucrose, vitamins (2.4 µM pyridoxine HCl, 1.4 µM nicotinic acid, 555 µM myoinositol, 1.2 µM thiamine HCl), Bactogar (7 g L^{-1} and 2.5 µM indolebutyric acid (IBA). Prior to autoclaving at 121 ºC and 106 kPa, the pH of media was adjusted to 5.6. 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ) was filter sterilized and added to the induction medium after autoclaving. After the placing the cotyledons on 10 cm diameter petri plates containing 30 mL of medium, the plates were wrapped by aluminums foils to provide darkness for the first ten days of the experiments. Then the explants were brought to light and were incubated at 24 ± 1 ºC under 16/8 h (light/dark) photoperiod with a Photosynthetic Photon Flux of about 50 µmol m^{-2} s^{-1}.

TDZ pretreatments: For the TDZ pre-treatment, the explants were imbibed for 24 h in sterile distilled water containing different levels of TDZ (0, 0.23, 0.69, 1.15 or 2.3 µM TDZ).

Elongation, rooting and acclimatization: The adventitious shoots formed on the cotyledons were excised and transferred on a shoot growth medium consisted of QL basal salts supplemented with 30 g L^{-1} sucrose, vitamins (2.4 µM pyridoxine HCl, 1.4 µM nicotinic acid, 555 µM myo-inositol, 1.2 µM thiamine HCl), Bactogar (7 g L^{-1} and 2.5 µM indolebutyric acid (IBA). The pH of the medium was adjusted to 5.6 and autoclaved as described above.

After a month of elongation, the shoots obtained from the growth medium were transferred to a rooting medium consisted of ½ strength MS[19] basal salts supplemented with the same vitamins as described above, 14.8 µM indolebutyric acid (IBA), 25 g L^{-1} sucrose and 7 g L^{-1} Bactogar. The pH of media was adjusted to 5.6 and autoclaved at 121 ºC and 106 kPa.

After rooting, the plantlets were transferred to soil in 8 cm × 8 cm pots and acclimatized by covering with a transparent lid in a culture room maintained at 22 ± 1 ºC under 16/8 h photoperiod. After 4 week of acclimatization period, the plants were transferred to a greenhouse.

Data analysis: All experiments were arranged in a completely randomized design and the data were collected 28 d after culture initiation. Following the arcsine square root transformation, the data was analyzed using (ANOVA) with mean separation by Duncan's; PROCGENMOD (SAS 9.1, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Effects of different standard basal salt compositions and TDZ pre-immersments on adventitious shoot regeneration from cotyledons of *P. avium* L. were assayed *in vitro* to optimize the shoot regeneration.

The cotyledon explants placed on the shoot induction medium started to expand and formed semi-hard callus in the dark. At the end of the dark incubation period, cultures were brought to light and they started to develop green colouring in the light. The adventitious shoots on the explants were clearly visible after 18 d when cultured onto WPM. On QL medium the shoots started to appear 20 d after the culture initiation. The shoot formation was slowest on the B5 medium and they only started to be visible after 22 d the culture initiation. The shoots developed on WPM and QL medium appeared to be healthier and greener than the shoots developed on the B5 medium. Red colouration along with adventitious shoot formation was also observed on the explants that cultured on WPM.

Vista cotyledons cultured on WPM and QL medium gave significantly higher regeneration frequencies than the explants that cultured on B5 medium

(Table-1). Although the regeneration frequency on WPM was also slightly higher than that on the QL medium, the difference was not statistically significant (Table-1). The basal media significantly effected average shoot number per regenerating explant. The highest shoot number per regenerating explant was obtained on WPM (4.3) followed by QL medium (3.1) (Table-1). Average shoot number per regenerating explant was significantly lower on B5 medium than WPM and QL medium.

TABLE-1
INFLUENCE OF DIFFERENT STANDARD BASAL SALT MIXTURES ON ADVENTITIOUS SHOOT FORMATION FROM COTYLEDONS OF *Prunus avium* CV. VISTA (n = 30)

Treatment	Regeneration*	Shoot number
WPM	67.0 a**	4.30 a
QL	57.0 a	3.10 ab
B5	10.0 b	2.25 b

*Presented as treatment means. Explant number with at least one shoot/total explants number x 100. **Significant differences by $p \leq 0.05$ by Duncan's test within the same column are presented by different letters.

TDZ pre-treatments did not enhance regeneration frequencies in any of the cultivars and in any of the concentrations tested. Moreover, some of the TDZ pre-treatments resulted in lower regeneration frequencies as compared to non-treated explants (Table-2). Although the regeneration frequencies were not improved by the TDZ pre-treatments, the average shoot number per regenerating explant were slightly increased when the cotyledon explants were imbibed in 2.3 µM TDZ in three of the cultivar tested (Table-2).

TABLE-2
EFFECTS OF THIDIAZURON PRE-TREATMENTS ON ADVENTITIOUS SHOOT REGENERATION FROM DARK INCUBATED COTYLEDONS OF SWEET CHERRY CULTIVARS (n = 30)

TDZ Pretreatment (µM)	Cherry cultivar					
	Vista		Heid		Tehranivee	
	RE*	SN	RE	SN	RE	SN
Control	53.3 a**	2.6 ± 0.3	16.6 a	1.5 ± 0.3	10.0 a	2 ± 0
0.23	20.0 c	2.0 ± 0.5	13.3 a	1.3 ± 0.3	–***	–
0.69	33.3 abc	2.9 ± 0.5	0.0 b	0	6.7 a	2 ± 0
1.15	29.0 bc	2.6 ± 0.8	13.3 a	1.3 ± 0.3	6.7 a	2 ± 1
2.3	50.0 ab	3.7 ± 0.6	10.0 a	2.3 ± 0.3	4.7 a	3 ± 0

*Number of explants with at least one shoot/total number of explants x 100. Data were recorded 28 days after culture initiation.

**Different letters in the same column denote significant differences at $p \leq 0.05$ by Duncan's test. ***Infected; RE = Regeneration; SN = Shoot number.

Shoot regeneration was highly depended on the basal media used. QL and WPM media stimulated shoot regeneration more than B5 medium and regeneration ability of the sweet cherry cotyledons was significantly reduced when B5 basal salt mixture was used in shoot induction medium. Similar findings were reported by other researchers where QL medium was better for stimulating shoot organogenesis in leaf explants and B5 medium caused significant reduction in regeneration ability[20]. QL medium was successfully used in promoting shoot regeneration from different explants of several species[15]. In agreement with present results, WPM medium was also optimal for organogenesis in leaf explants of several *Prunus* species[12,13,21]. WPM was developed for wood plant species[17] and QL medium was especially developed for *Prunus* species[16]. Both have lower macro salts content than MS medium, in addition both have lower levels of NH_4^+ concentrations. Ammonium ions can be toxic to plant cell cultures and many researchers used low concentrations of ammonium because of its toxicity[22]. The improved regeneration frequencies observed on WPM and QL media might be associated with their low nitrogen level and salt content.

Short term and low concentration pretreatments of explants with TDZ, a cytokinin-like phenylurea derivative, has been found to enhance shoot regeneration in several species[10,23]. In contrast with these results, TDZ pretreatments were found to be in effective in increasing regeneration frequencies from cherry cotyledons in our study. Therefore, the effect of TDZ pre-treatment might be specific to explant type and species.

Most of the current transformation protocols in *Prunus* species developed using seed material. Unlike immature embryos, stored seed materials are suitable to use year-round in genetic engineering studies[9] to achieve different objectives in basic research fields such as gene silencing and expression studies, promoter and marker gene assessments and resistance assessments to pests.

In conclusion, it is reported that basal salt composition is an important factor and significantly influence regeneration from cherry cotyledons. The importance of genetic transformation in fruit breeding is increasing due to the extensive breeding time required for conventional methods. Availability of a reliable and efficient regeneration protocol is must to develop a genetic transformation technology for sweet cherry, therefore, high frequency regeneration of cherries from cotyledons may play an important role for cherry breeding in the future.

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