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Establishment of *In vitro* Adventitious Root Cultures and Analysis of Flavonoids in *Rumex crispus*

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Abstract

Adventitious root culture of leaf explants of *R. crispus* was established using different MS supplemented with different concentrations of auxins and a combination of NAA and Kn for growth and flavonoids production. Among the different auxins, NAA was more effective than IAA to induce adventitious roots. Adventitious roots grown on MS containing 5 μM NAA and 0.5 μM Kn showed the highest root growth, as well as the highest amount of total flavonoids (= **6**) as compared with roots grown in other media. Chromatographic purification of the root extract showed that flavonoid composition also was influenced by hormone combinations in the culture media. The addition of Kn to the medium reduced or suppressed myricetin (M) and naringenin (N) production. Quercetin (Q) was not found in media containing Kn alone similar to the control medium. Isorhamnetin (I), kaempferol (K) and rutin (R) were produced in the roots on media supplemented with all hormone combinations, but were absent in 0.1 μM Kn supplemented media similar to the control roots.

Introduction

Rumex crispus is an herbaceous perennial weed plant, belongs to the family Polygonaceae. The species is distributed widely in the humid regions of the northern hemisphere, mostly in the acidic (silicate) soils of Iran. Traditionally, its roots have been largely recommended by herbalists for a wide range of skin diseases (El-Bakry et al. 2012).

The plant species contains many bioactive substances such as flavonoids, anthraquinones particularly in roots, tannins, saponins and triterepenoids (Noori et al. 2009, Rao et al. 2011, Orban-Gyapai et al. 2014).

Flavonoids possess a wide range of biological activities, medicinal and pharmacological effects (Noori 2012).

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Plant cell culture technologies developed in the past as possible tools for production of secondary metabolites. Secondary metabolites production is under strict metabolic regulation and tissue specific localization. Hence the differentiated cultures such as root cultures are widely studied. Roots of numerous plant families are the site of biosynthesis or accumulation of major secondary metabolites. *In vitr*o root culture has become an alternative method for the production of valuable secondary metabolites on a commercial scale (Chandra and Chandra 2011).

Adventitious roots induced by *in vitro* methods showed a high rate of proliferation and are the site of active secondary metabolism (Hahn et al. 2003, Yu et al. 2005). Adventitious roots are natural, grow vigorously in phytohormone supplemented medium and have shown tremendous potentialities of accumulation of valuable secondary metabolites (Murthy et al. 2008). It was reported that adventitious root induction technique has been applied on economically important plants (Rowinsy et al. 1990, Tomoyoshi et al. 2005). However, optimization and scale-up is required to increase root biomass and secondary metabolites (Choi et al. 2000, Yu et al. 2001, Kim et al. 2004, Lee et al. 2006).

Therefore, the present of the study has been undertaken to develop an efficient protocol *in vitro* root cultures from leaf explants of *R. crispus* and to investigate the effects of auxins and cytokinins on flavonoid contents of *in vitro* grown adventitious roots.

Materials and Methods

In vitro cultured plantlets of *Rumex crispus* were established from seeds on basal MS and were used as plant material in this study. Leaves which were excised from *in vitro* grown plantlets at the age of 7 weeks, cut into small segments (0.7 cm × 0.7 cm) and cultured on MS (3% sucrose) supplemented with different concentrations (0, 2.5, 5 and 10 μM) of IAA or NAA. The second experiment involved combining 2.5 or 5 μM NAA with different concentrations of Kn (0, 0.1, 0.5 μM) or 3% (w/v) coconut milk (CM). All the cultures were maintained for 3 or 4 weeks at 25 ± 1°C in the dark. The rooting percentage, fresh weight (mg), root length (cm) and the number of roots formed per explant were recorded after 3 or 4 weeks.

A ground-dried root (0.2 g) was refluxed with 10 ml of 80% methanol (MeOH) at 80°C for 1 hr. The mixture was cooled and therefter centrifuged at 4000 g for 10 min, the supernatant solution was filtered. The extract was evaporated to dryness by rotary evaporation at 40°C and taken up in 2 ml of 80% MeOH for analysis by 2-dimensional paper chromatography (2D PC) and thin

layer chromatography (TLC) based on available references (Mabry et al. 1970, Markham 1982).

For the detection of flavonoids, ca. 20 µl of each extract was applied to chromatography paper as a concentrated spot (10 applications of 20 µl). The chromatogram for each sample was developed in BAW (n-BuOH-HOAc-H_2O = 4 : 1 : 5; v/v) for first direction and in HOAc (= 15% aqueous acetic acid) for second direction. After development, the chromatograms were viewed in long wave UV light (366 nm) and fluorescent spots were marked. Rf values in BAW and 15% HOAc were calculated.

Purified flavonoids were identified by means of UV spectrophotomerty using Schiff reagents to investigate the substitution patterns of the flavonoids (Mabry et al. 1970, Markham 1982) and by acid hydrolysis to identify the aglycone and sugar moieties. The TLC plates were run in three solvents alongside standards to identify the aglycone moiety (Harborne 1998, Narayan et al. 2005). Co-chromatography with standards was also performed where possible. Flavonoid standards available for comparison during the study were obtained commercially from Merck and Sigma.

Data were analyzed by one-way ANOVA to detect significant differences between means. Means differing significantly were compared using the DMRT at the 5% probability level.

Results and Discussion

Authors tested various explants including root, stem, petiole and leaf segments to find the best explant to adventitious root initiation. Adventitious root cultures were not established by the root (Fig. 1a) and stem explants, because of slow growth and development of adventitious roots from both the explants. Thus, the leaf and petiole explants were alternatively used to establish the root culture of curled dock plant. Adventitious roots were fully developed from the leaf explants within 3 weeks (Fig. 1b) followed by petiole explants (4 weeks) (Fig. 1c). So leaf explants was found to be the best explants in root induction.

Auxins promote the formation of adventitious roots in explants. Among the NAA concentrations, supplementation of 2.5 µM NAA to culture medium generated the highest rooting percentage (65) within 3 weeks, followed by 5 µM NAA (55). Contrarily, root initiation was suppressed in 10 µM NAA (Table 1). The highest root number per explant was achieved in 2.5 µM NAA within 3 weeks (Fig. 2a).

IAA was also found to successfully induce the formation of adventitious roots, and the highest rooting ability for IAA was achieved in 10 µM

supplemented with 10 μM IAA within 4 weeks (Table 1, Fig. 2b). Contrary to present authors' result, in *Malus* (Klerk et al. 1997) IAA was effective for the production of roots and NAA strongly inhibited the growth of roots. This is in line with the fact that a particular type of auxin is effective in enhancing rooting in particular species (Puri and Shamet 1988). Thus authors selected 2.5 and 5 μM concentrations of NAA for further experiments on effects of cytokinins in combination with NAA on rooting and root biomass production in explants.

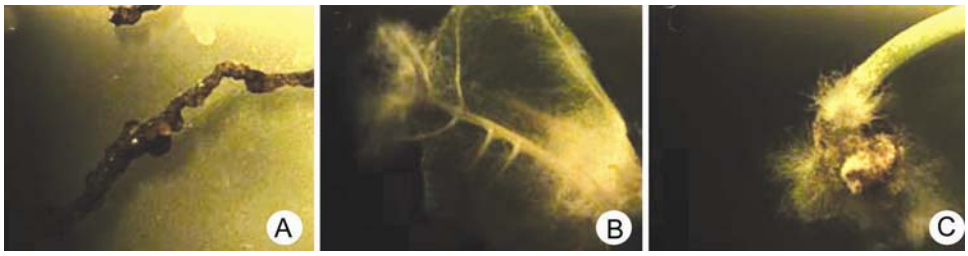

Fig. 1. The effects of NAA (5 μM) on the formation of adventitious roots on the segments of *R. crisps* in 3 - 4 weeks. **A.** Smaller no. of roots were seen on the surface of the main root. B. Formation **of** highly branched roots on leaf explants. **C.** Thin and short roots on the surface of the petiole.

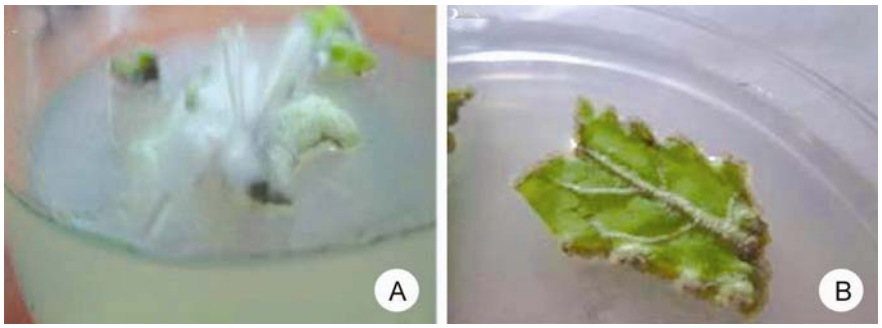

Fig. 2. Adventitious root formation from *in vitro* leaf explants in the medium containing either: (A) 2.5 μM NAA or (B) 10 μM IAA.

The influence of NAA alone on root growth (fresh weight) was negligible. The addition of Kn (0.5 μM) significantly increased root weight in treatments containing NAA, which indicates that Kn could promote the growth of the roots, however the root number and rooting percentage was decreased in MS containing Kn. Narayan et al. (2005) and Lee (2009) also reported that a combination of cytokinin and high auxin levels increased biomassin cell cultures of *Daucus carota* and *Eleutherococcus koreanum*, respectively.

However, upon addition of Kn alone at high concentration (0.5 μM) no adventitious roots were formed and only abnormal roots with thicker and

elongated tips were observed. The highest number of roots and rooting percentage were obtained in leaf explants cultured on MS with 5 μM NAA and 5 μM NAA + 3% CM (coconut milk) (Table 2). 0.5 μM Kn and 3% CM in combination of 5 μM NAA increased root weight (Table 2).

Table 1. The effect of different concentrations of NAA and IAA in the rooting of *R. crispus* leaf explants cultured on MS. Values are means of 6 replicates ± S.E. Means followed by the same letter are not significantly different at $p < 0.05$ according to DMRT.

Hormonal treatment	% rooting	No. of roots/explant	Days of root initiation
Control	0^{c}	0^{d}	0
2.5 μM NAA	65 ± 5^{a}	4.5 ± 0.2^{a}	21
5 μM NAA	55 ± 2.4^{a}	5.1 ± 0.2^{a}	21
10 μM NAA	33.3 ± 3.3^{b}	2 ± 0.6^{bc}	21
2.5 μM IAA	53 ± 4.7^{a}	1.3 ± 0.3^{c}	28
5 μM IAA	60 ± 5^{a}	2.5 ± 0.5^{bc}	28
10 μM IAA	60 ± 2.9^{a}	3 ± 0.6^{b}	28

Table 2. The effect of NAA, Kn and CM (coconut milk) on rooting, number of roots per explant, root length (cm) and root fresh weight (mg) of *R. crispus* cultured on MS. Values are means of 6 replicates ± S.E. means followed by the same letter are not significantly different at $p < 0.05$ according to DMRT.

Hormonal treatment	% rooting	No. of roots/ explant	Root length	Fresh weight of roots
Control	0^{d}	0^{f}	0^{d}	0^{d}
0.1 μM Kn	0^{d}	0^{f}	0^{d}	0^{d}
0.5 μM Kn	20.3 ± 1.7^{c}	1.6 ± 0.4^{ef}	1.5 ± 0.2^{a}	8.1 ± 0.5^{a}
2.5 μM NAA	50.8 ± 2.7^{a}	3.8 ± 0.6^{cd}	0.5 ± 0.07^{bc}	1.3 ± 0.3^{d}
2.5 μM NAA + 0.1 μM Kn	50 ± 0^{a}	4 ± 0^{cd}	0.5 ± 0.05^{bc}	1.5 ± 0.5^{d}
2.5 μM NAA + 0.5 μM Kn	20 ± 0^{c}	1.5 ± 0.5^{ef}	0.3 ± 0.05^{cd}	3.7 ± 0^{c}
5 μM NAA	47.8 ± 1.4^{a}	5.8 ± 0.4^{ab}	0.9 ± 0.08^{b}	1.5 ± 0.2^{d}
5 μM NAA + 0.1 μM Kn	46.9 ± 2.4^{a}	4.2 ± 0.3^{bc}	0.8 ± 0.2^{b}	1.8 ± 0.4^{d}
5 μM NAA + 0.5 μM Kn	35 ± 3.1^{b}	3 ± 0.3^{cde}	0.7 ± 0.1^{b}	3.9 ± 0.9^{bc}
5 μM NAA + CM (3 %)	45 ± 3.5^{a}	6.8 ± 0.5^{a}	0.8 ± 0.2^{b}	5.8 ± 1^{b}
5 μM NAA + CM (3%) + 0.5 μM Kn	45.8 ± 2.8^{a}	2.3 ± 0.6^{de}	0.6 ± 0.09^{bc}	5.1 ± 0.8^{bc}

Coconut milk contains a kind of cytokinins called zeatin and increase root weight. Effect of cytokinins in combination with NAA on rooting depends on kind and concentration of cytokinin. Panichayupakaranant and Tewtrakul (2002) reported that the combination of NAA and Kn was found to be best for root growth in *P. rosea*. However, in the present study, the combination of NAA and Kn was not suitable for increasing the rooting percentage.

The type and concentration of auxin and cytokinin, either alone or in combination, has been known to strongly influence growth as well as the secondary metabolites in tissue culture. Flavonoids were isolated and identified by 2D paper and thin layer chromatography. Results of total flavonoids (Table 3) showed that maximum number of total flavonoids (= 6) was found in roots on media containing 2.5 μM NAA alone or in combination of Kn followed by roots obtained on 5 μM NAA. Addition of 0.5 μM Kn showed a beneficial effect for biomass, as well as flavonoid production. Addition of auxin (2, 4-D) and Kn into the media was also found to enhance the flavonoids production in *Genista tinctoria* (Luczkiewics and Glod 2003). According to Maurmann et al. (2006), the presence of Kn in combination with 2,4-D was beneficial to valeportiate accumulation in *Valeriana gelechomifolia* callus culture.

Table 3. Flavonoid screen of adventitious roots formed on leaf explants cultured in different media by 2-dimensional paper and thin layer chromatography.

Treatment	NTF	FSN	FCN	AN	I	K	MY	N	Q	RU	V
0 μM NAA + 0 μM Kn	0	0	0	0	–	–	–	–	–	–	–
0 μM NAA + 0.1 μM Kn	0	0	0	0	–	–	–	–	–	–	–
0 μM NAA + 0.5 μM Kn	3	1	1	1	+	+	±	+	–	+	–
2.5 μM NAA + 0 μM Kn	6	2	3	1	+	+	+	+	+	+	±
2.5 μM NAA + 0.1 μM Kn	6	2	2	2	+	+	–	–	+	+	–
2.5 μM NAA + 0.5 μM Kn	6	2	2	2	+	+	–	–	+	+	–
5 μM NAA + 0 μM Kn	4	2	1	1	+	+	+	+	+	+	–
5μM NAA + 0.1 μM Kn	6	2	2	2	+	+	±	–	+	+	–
5μM NAA + 0.5 μM Kn	6	2	2	2	+	+	±	–	+	+	–

NTF = Number of total flavonoids. FSN = Number of flavonoid sulphates. FCN = Number of flavone C- and C-/O-glucosides. AN = Number of aglycones. I = Isorhamnetin, K = Kaempferol. MY = Myricetin. N = Naringenin. Q = Quercetin. RU = Rutin. V = Vitexin. Scored characters: – Flavonoid absent. ± Flavonoid rare. + Flavonoid present.

Roots obtained on 0.5 μM Kn were found to contain the least amount of total flavonoids (= 3) and in media without plant hormones (control) and or low concentration of Kn, flavonoids were not found (Table 3).

The flavonoid composition was also influenced by the hormonal combinations in the culture media. The addition of Kn to the medium either reduced or suppressed the production of myricetin and naringenin (Table 3). Quercetin was not found in the media containing kinetin alone as in the control medium. Isorhamnetin, kaempferol and rutin were produced in the roots

cultured in the with all the hormonal combinations but were absent in 0.1 μM Kn and also in the control roots (Table 3).

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