Studies of *in vitro* Adventitious Root Induction and Flavonoid Profiles in *Rumex crispus*

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Abstract   Flavonoids are the principal bioactive components of the medicinal plant *Rumex crispus*, to which various diverse pharmacological properties are attributed. Adventitious root culture of leaf explants of *R. crispus* was established using different MS media supplemented by different concentrations of Auxins and a combination of Naphthalene Acetic Acid (NAA) and Kinetin (Kn) for growth and flavonoids production. NAA was more effective than Indole Acetic Acid (IAA) to induce adventitious roots. 5 µM NAA treatments had the most number of roots. Maximum root length and root fresh weight were obtained in 0.5 µM Kn. 0.5 µM Kn using in combination with NAA in culture medium increased root fresh weight. Results of 2-dimensional paper and thin layer chromatography of the samples extracts showed that hormons are influenced flavonoids in culture medium. The combination of NAA and Kinetin in culture medium reduced or suppressed Myricetin and Naringenin production and increased number of aglycones. Quercetin was not found in root on medium containing 0.5 µM Kn. Isorhamnetin, Kaempferol, Rhamnetin and Rutin were found in all of treatments that had produced roots. It is believed that root flavonoids are related to root growth, development and differentiation in *R. Crispus* species.

Keywords   Adventitious root, Auxin, Cytokinin, Flavonoids, *Rumex crispus*

1. Introduction

Rumex crispus is an herbaceous perennial weed plant, belongs to Polygonaceae which has been distributed widely in the humid regions of northern hemisphere including Iran, mostly in the acidic (silicate) soils. Traditionally, its roots have been largely recommended by herbalists for range of skin diseases [1]. The plant contains many bioactive substances such as flavonoids, anthraquinones (particularly in roots), tannins, saponins and triterepenoids [2-4]. Flavonoids possess a wide range of biological activities, medicinal and pharmacological effects [5].

Plant cell culture technologies developed in the past as possible tools for secondary metabolites production. In most cases, for the large scale production of these compounds, production is too low for commercialization. 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 for 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 commercial scale [6].

Transformed root (hairy root) cultures produced by *Agrobacterium rhizogenesis* offer a promising alternative method to cell culture, but hairy roots usually produce opine-like substances which are lethal to mammalian cells [7] and also GMOs are not always accepted in several countries like Australia, Japan, France, Korea, New Zealand etc. Adventitious roots induced by *in vitro* methods showed high rate of proliferation and active secondary metabolism [8, 9]. Adventitious roots are natural, grown vigorously in phytohormone supplemented medium and have shown tremendous potentialities of accumulation of valuable secondary metabolites [10]. It was reported that adventitious root induction technique has been applied on economically important plants [11, 12]. For example, mass production of isoflavonoids through adventitious root cultures of *Iris germanica* had been conducted by Tomoyoshi et al (2005) who noticed that its adventitious root cultures accumulated a high concentration of isoflavones that capable to be utilized in medicinal production [12]. However, optimization and scale-up is required to increase root biomass and secondary metabolites [13-16].

Therefore, the purpose of the study was to develop an efficient *in vitro* development of root cultures from leaf explants of *Rumex crispus*, an important medicinal plant. In the present work, we have established a reproducible method for high frequency root induction, root growth from leaf explants on MS solid medium and investigated the effects of Auxin and Cytokinin on flavonoid constitutes of *in vitro*

adventitious roots.

2. Materials and Methods

2.1. Plant Material and Adventitious Root Culture Establishment

In vitro cultured plantlets of *Rumex crispus* were established from seeds (collected from Arak, Iran, 34° 06′ N, 49° 46′ E during 2013) on basal Murashige and Skoog (MS) medium [17] and were used as plant material in this study. Leaves which were excised from *in vitro* grown plantlets at the age of 8 weeks, cut into small segments (0.7 cm×0.7 cm) and cultured on MS medium (3% sucrose) supplemented with different concentrations (0, 2.5, 5 and 10 µM) of Indole-3-Acetic Acid (IAA) or 1-Naphthalene Acetic Acid (NAA). The second experiment involved combining 2.5 or 5 µM NAA with different concentrations of Kinetin (0, 0.1, 0.5 µM). All the cultures were maintained for 3 weeks at 25 ± 1°C in dark. The rooting percentage (%), fresh weight (mg), root length (cm) and the number of roots formed per explant were recorded after 3 weeks.

2.2. Preparation of Root Extract

A ground-dried root (0.2 g) was refluxed with 10 ml of 80% methanol (MeOH) at 80 °C for 1 h. The mixture was cooled and after centrifugation at 4000g for 10 min, the supernatant solution was filtered. The extract 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 [18, 19].

2.3. Isolation and Identification of Flavonoids by 2-DPC

For the detection of flavonoids, ca 20 µl of each extracts 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.

2.4. Identification of Flavonoids Types

When sufficient amounts of purified flavonoids had been obtained, they were identified by means of UV spectrophotomerty using shift reagents to investigate the substitution patterns of the flavonoids [18, 19] 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 [20, 21]. Co-chromatography with standards was also performed where possible. Flavonoid standards available for comparison during the study obtained commercially from Merck, Sigma and Fluka).

2.5. Statistical Analysis

Data were analyzed by One-way analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using the Duncan's multiple range tests at the 5% probability level.

3. Results and Discussion

3.1. Root Formation from Different Explants

We 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 (Figure 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 2 weeks (Figure 1b) followed by petiole explants (3 weeks) (Figure 1c). So leaf explants was found to be the best explants in root induction.
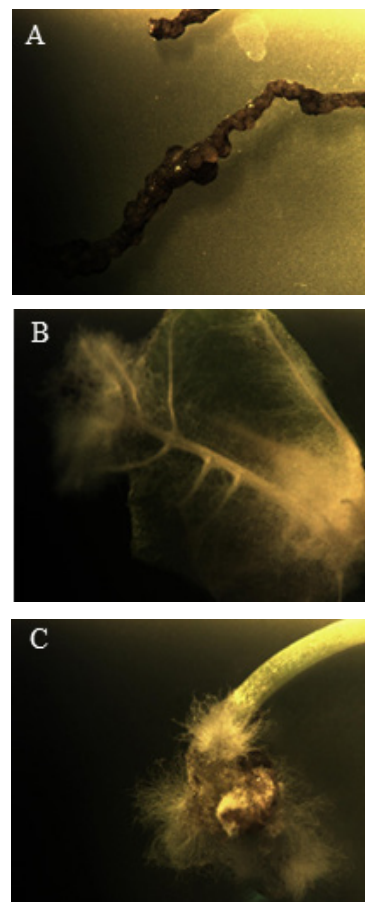

Figure 1. The effects of NAA (5µM) on the formation of adventitious roots on the segments of *Rumex crispus* within 2-3 weeks. **A.** Less roots occurred at the main root, **B.** Highly branched roots formation on leaf explants, **C.** Thin and short roots in petioles

3.2. Effect of Exogenous Auxins and Cytokinin on Root Production

Auxins promote the formation of adventitious roots on explants. Among the NAA concentrations, supplementation of 2.5 µM NAA to culture medium was generated the highest rooting percentage (65%) within 2 weeks, which was 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 5 µM NAA within 2 weeks. IAA also found to be successfully induced the formation of adventitious roots, and the highest rooting ability for IAA was achieved in 10 µM within 3 weeks (Table 1). Contrary to our result, in *Malus* [22] 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 [23]. Thus we selected 2.5 and 5 µM concentrations of NAA for further experiments on effects of Cytokinin in combination with NAA on rooting and root biomass production in explants. The highest number of roots and rooting percentage were obtained in leaf explants cultured on MS media with 5 µM NAA, but by the reason very low root weight, this medium was not suitable for rooting (Table 2, Figure 2a). The influence of NAA alone on root growth (fresh weight) was negligible. The addition of Kinetin (0.5 µM) significantly increase root weight in treatments containing NAA, which means Kinetin could promote the growth of the roots, however the root number was decreased in MS media containing 0.5 µM Kn.

Table 1. Effect of different concentrations of NAA and IAA on rooting of *Rumex crispus* leaf explants cultured on MS medium. Values are means of 6 replicates ± S.E. Means followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range tests

Hormonal treatment	% rooting	No. of roots/explant	Days of root initiation
Control	0 [C]	0 [e]	0
2.5 µM NAA	65 ± 5 [a]	3.5 ± 0.2 [b]	14
5 µM NAA	55 ± 2.4 [a]	5.1 ± 0.2 [a]	14
10 µM NAA	33.3 ± 3.3 [b]	2 ± 0.6 [cd]	14
2.5 µM IAA	53 ± 4.7 [a]	1.3 ± 0.3 [d]	21
5 µM IAA	60 ± 5 [a]	2.5 ± 0.5 [bc]	21
10 µM IAA	60 ± 2.9 [a]	3.5 ± 0.6 [b]	21

Narayan et al. (2005) and Lee (2009) reported that a combination of cytokinin and high Auxin levels increased biomassin cell cultures of *Daucus carota* and *Eleutherococcus koreanum*, respectively [21, 24]. Maximum root length and root fresh weight was observed on MS medium containing 0.5 µM Kinetin, but by the reason very low rooting percentage and root number, this medium was not suitable for rooting (Table 2, Figure 2b). Also Hill et al (1989) reported some examples of rooting stimulation using Cytokinin without combination of Auxins [25]. 0.5 µM Kn in combination of NAA increased root weight (Table 2). Effect of Kn in combination with NAA on rooting depends

on concentration. Finally 5 µM NAA + 0.5 µM Kn treatment was the best culture medium for rooting and also this treatment had the most flavonoid number (Table 2, 3). Panichayupakaranant and Tewtrakul (2002) reported that the combination of NAA and Kn was found to be best for root growth in *Plumbago rosea* [26].
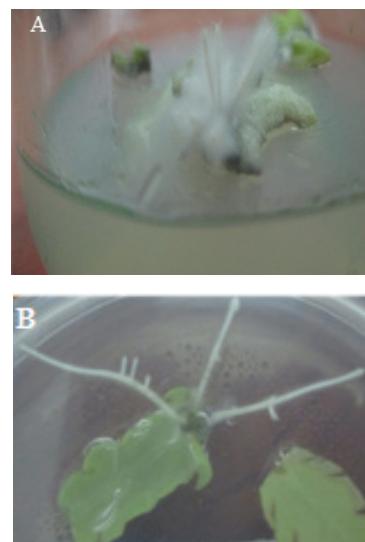

Figure 2. Adventitious root formation from *in vitro* leaf explants in the medium containing: **A.** 5 µM NAA or **B.** 0.5 µM Kn

3.3. Effect of Exogenous Auxin and Cytokinin on Flavonoids Production

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 2-dimensional 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 Kinetin 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 were also found to enhance the flavonoids production in *Genista tinctoria* [27]. According to Maurmann et al (2006), the presence of Kn in combination with 2, 4-D was beneficial to valepotriates accumulation in *Valeriana gelechomifolia* callus culture [28]. Roots obtained on 0.5 µM Kn were found to contain the least amount of total flavonoids (=3). The flavonoid composition also was influenced by hormone combinations in culture media. The combination of NAA and Kinetin in culture medium reduced or suppressed Myricetin (My) and Naringenin (N) production and increased number of aglycones. Quercetin was not found in root on medium containing 0.5 µM Kn. Isorhamnetin, Kaempferol, Rhamnetin and Rutin were found in all of treatments that had produced roots. There was not Vitexin in all of roots with the exception of 2.5 µM NAA treatment (Table 3). All of studied samples lacked Apigenin, Chrysin, Genistein, Luteolin, Morin and Tricin.

Table 2. Effect of NAA and Kn on rooting percentage, number of roots per explant, root length (cm) and root fresh weight (mg) of *Rumex crispus* cultured on MS medium. Values are means of 6 replicates ± S.E. Means followed by the same letter are not significantly different at *P < 0.05* according to Duncan's multiple range tests

Hormonal treatment	% rooting	No. of roots/explant	Root length	Root fresh weight
Control	0 [c]	0[d]	0[d]	0[c]
0.1 µM Kn	0 [c]	0[d]	0[d]	0[c]
0.5 µM Kn	20.3 ± 1.7 [b]	1.6 ± 0.4 [cd]	1.5 ± 0.2 [a]	8.8 ± 0.5 [a]
2.5 µM NAA	50.8 ± 2.7 [a]	3.8 ± 0.6 [b]	0.5 ± 0.07 [bc]	2 ± 0.3 [c]
2.5 µM NAA+0.1 µM Kn	50 ± 0 [a]	4 ± 0 [b]	0.5 ± 0.05 [bc]	2.2 ± 0.5 [c]
2.5 µM NAA+0.5 µM Kn	20 ± 0 [b]	1.5 ± 0.5 [cd]	0.3 ± 0.05 [cd]	4.4 ± 0 [b]
5 µM NAA	50 ± 1.4 [a]	5.8 ± 0.4 [a]	0.9 ± 0.08 [b]	2.2 ± 0.2 [c]
5 µM NAA+0.1 µM Kn	46.9 ± 2.4 [a]	4.2 ± 0.3 [ab]	0.8 ± 0.2 [b]	2.5 ± 0.4 [c]
5 µM NAA+0.5 µM Kn	45 ± 3.1 [a]	3 ± 0.3 [bc]	0.7 ± 0.1 [b]	4.6 ± 0.9 [b]

Table 3. Flavonoid screens of adventitious roots formed on *Rumex crispus* leaf explants cultured on different media by 2-dimensional paper and thin layer chromatography

Treatment	NTF	FSN	FCN	AN	I	K	MY	N	Q	Rh	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	+	+	±	-	+	+	+	-

Abbreviations: NTF=number of total flavonoids, FSN=flavonoid sulphate number, FCN=flavone *C*-and *C*-/*O*-glucosides number, AN=aglycones number, I=Isorhamnetin, K=Kaempferol, My=Myricetin, N=Naringenin, Q=Quercetin, Rh=Rhamnetin, Ru=Rutin, V=Vitexin.

Scored characters: - (non flavonoid), ± (few flavonoid), + (middle concentration of flavonoid).

As Curir et al (1990) showed in reproduced *Eucalyptus gunnii* Hook plants from lateral bud stimulation *in vitro* method, flavonoids association and adventitious root production have direct relationship together and both are overshadowed hormonal treatment [29]. It is believed that root flavonoids are related to root growth, development and differentiation in this species.

4. Conclusions

This study has revealed the potential of *in vitro* adventitious roots as a source for flavonoids production in this species. The type and concentration of Auxin and Cytokinin, either alone or in combination strongly influence growth as well as the secondary metabolites in tissue culture. For example NAA was more effective than IAA to induce adventitious roots in *R. crispus*. It is believed that root flavonoids are related to root growth, development and differentiation in this species.

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