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Effect of Growth Regulators on Micropropagation, Callus Induction and Callus Flavonoid Content of *Rumex pictus* Forssk

Nadia Mohamed El-Shafey[#], Mona Sayed, Enas Shabaan Ahmed, Ola Hammouda and Salah El-Din Ali Khodary

Department of Botany and Microbiology, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.

THE CURRENT study investigates the effect of growth regulators on micropropagation, callus induction and callus flavonoid content of *Rumex pictus* Forssk; an endangered medicinal plant. Various combinations of kintin (KIN), 6-benzylaminopurine (BAP), Thidiazuron (TDZ) and indole-3-butyric acid (IBA) were used for micropropagation from shoot tip explants. Murashige and Skoog (MS) medium containing 2mg L^{-1} BAP was the best for micropropagation where shoot formation frequency was 70% with 12.6 shoots/explant. The highest root formation frequency resulted on half-strength MS medium fortified with 2mg L^{-1} naphthalene acetic acid (NAA) or 1mg L^{-1} IBA. One mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) combined with 0.4mg L^{-1} BAP induced the highest callus mass production from the investigated explants, particularly 1st foliage leaf that was the best explant for callus induction and proliferation. The accumulation of flavonoids in callus was diminished by BAP but enhanced by increasing 2,4-D concentration in culture medium. To our knowledge, this is the first report of micropropagation and callus induction of this endangered medicinal plant which will help in its rapid mass production, conservation and *in vitro* production of secondary metabolites.

Keywords: Auxins, Callus, Cytokinins, Dock, Flavonoids, Shoot induction.

Introduction

The genus *Rumex* belongs to family Polygonaceae. Many species of *Rumex* have shown a broad spectrum antimicrobial and antioxidant activity due to their phytoconstituents such as flavonoids, anthraquinones and terpenoids, which give the species potentiality to be used in folk medicine. Moreover, important bioactive substances such as vitexin, isovitexin, orientin, isorientin, emodin and chrysophanol were isolated from some of *Rumex* species (Batanouny et al., 1999 and Sayed et al., 2017). *Rumex pictus* Forssk is a winter annual herb that is commonly known as dock. It is used in folk medicine as sedative, spasmogenic and antimicrobial (Mossa et al., 2000). Batanouny et al. (1999) considered *R. pictus* as an endangered medicinal plant in Egypt. This species was also reported as endemic and rare in northern and eastern deep sand areas in Saudi Arabia (Rahman et al., 2004).

Plant tissue culture is considered an alternative method to the conventional propagation of plants. *In vitro* culture and micropropagation as biotechnological techniques play an important role in *ex situ* conservation of the endangered medicinal plants (Goyal et al., 2014; Mahendran & Narmatha, 2014 and Manole-Paunescu, 2014). Micropropagation provides all the year-round disease free plantlets production and rapid mass cloning of genotypes. In addition, it is an efficient mean of germplasm exchange, storage and maintenance. Micropropagation via shoot tips and stem meristems has been applied on rare and endangered medicinal plants like *Polygonum hydropiper, R. vesicarius, Hypericum gaitii* and *Rheum emodi* (Hasan & Sikdar, 2010; Abo El-Soud et al., 2011; Swain et al., 2016 and Tabin et al., 2017) due to preserving the genetic integrity of the propagated plants. Extensive studies investigated the utilization of plant cell cultures for the production of plant metabolites with pharmacological activities (Matkowski, 2008).

[#]Corresponding author emails: nadia69shafey@yahoo.com; nadia.elshafey@science.bsu.edu.eg
Tel.: +201014243923, Fax: 002-082-2334551
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Secondary metabolites production via callus culture has advantage over their production from the intact plants, since the *in vitro* production is running under controlled conditions, independently from climatic and seasonal factors and provides a non-depleted and reliable source of natural products.

A main factor that masters the growth and success of the *in vitro* plant culture is growth regulators; auxins and cytokinins. The cytokinin, 6-benzylaminopurine (BAP), was found to induce *in vitro* regeneration of medicinal plants either alone (Al Khateeb et al., 2017) or when it was combined with auxins such as IBA (Tabin et al., 2017), NAA (Swain et al., 2016) or IAA; indole-3-acetic acid (Moharami et al., 2014 and Ślesak et al., 2014). Moreover, the involvement of cytokinins in callus culture media and their contribution in callus induction and proliferation are well established. Flavonoids are one of the largest groups of secondary metabolites that recently have been the subject of considerable scientific and therapeutic interest due to their proven ability to scavenge reactive oxygen species (ROS) in general (Matkowski, 2008). Plant growth regulators were found to affect the accumulation of flavonoids in cell culture (Murthy et al., 2014; Ji et al., 2015 and El-Shafey et al., 2016). The significant role of growth regulators on the biosynthesis of flavonoids is well documented, although it is not yet known how they act on the biosynthetic pathways (Jamwal et al., 2018). Combination and concentration of the applied auxin and cytokinine were reported to strongly influence the biosynthesis and accumulation of flavonoids in the cultured plant cell (Bota & Deliu, 2015). Plant tissue culture techniques have been applied to some *Rumex* species other than *R. pictus* such as *R. vesicarius* (Abo El-Soud et al., 2011; El-Shafey et al., 2016 and Sayed et al., 2017), the hybrid sorrel *Rumex tianschanicus* x *Rumex patientia* (Ślesak et al., 2014), *R cyprius* (Al Khateeb et al., 2017) and *R. nepalensis* (Bhattacharyya et al., 2017). However, to our knowledge, there have been no previous reports investigating the *in vitro* culture of *R. pictus*.

This work studied the effect of growth regulators on micropropagation of *R. pictus* from shoot apex explant. Effect of growth regulators on the production of *R. pictus* callus and accumulation of its flavonoids was also investigated.

Materials and Methods

Plant material and seed germination

Rumex pictus Forssk seeds were collected from the Mediterranean coastal region, Alexandria, Egypt in April 2011 and the plant species was identified according to Bolous (1999). Seeds were surface sterilized using 70% ethanol for 30sec followed by 40% commercial bleach (5% NaOCl) for 15min. The seeds were rinsed three times in sterile distilled water and germinated on MS medium (Murashige & Skoog, 1962) supplemented with 8g L^{-1} agar. Cold stratification was applied to the cultured seeds by incubating at 4ºC for 4 days to induce germination. After radical emergence, cultured seeds were transferred to growth chamber and incubated at 25±2ºC under (16/8h) light/dark photoperiod using white cool fluorescent light (100µmol m^{-2} s^{-1}).

Shoot proliferation and multiplication

Shoot apices (4-5mm in length) were excised from 30 days-old seedlings and transferred to a baby food jar containing 20ml of medium. Various media were investigated for shoot induction; each was prepared by supplementing MS medium with 30g L^{-1} sucrose and growth regulators (BAP, KIN and TDZ, either alone or in combination with IBA; Table 1), while MS growth regulators-free medium was used as control. The pH of the medium was adjusted at 5.8 and 8g L^{-1} agar were added prior to autoclaving for 20min at 121ºC and 15psi pressure. The cultures were incubated at 25±2ºC under (16/8h) light/dark photoperiod. Explants were sub-cultured once after 4 weeks.

In vitro root formation and acclimatization

Shoots of 3-4cm length were separated and transferred to MS media (full-strength or half-strength) supplemented with 20g L^{-1} sucrose. Growth regulators were supplemented as 1mg L^{-1} IAA, 1 and 2mg L^{-1} of IBA and NAA, individually. Media were solidified by 2.5g L^{-1} phytagel (Duchefa, Haarlem, The Netherlands), where roots were not induced on media solidified with agar. After 3 weeks of root induction, the plants with well developed roots were washed gently under running tap water and treated with fungicide (1mg L^{-1} Metalaxyl™, the national company for agrochemicals production, Egypt) solution. The plants were transferred to paper cups (6cm diameter) containing sterile peat moss soil. The plants were covered with transparent polyethylene bags, to maintain humidity and

allow penetration of light, and incubated in the growth chamber at 25±2ºC under (16/8h) light/ dark photoperiod. Plants were irrigated using half-strength MS salts and gradually acclimatized to low humidity by making a hole on the plastic bag and increasing its size every 3 days, until establishment of the plants in the soil and formation of new leaves. After 2 weeks, the plants were uncovered and maintained in the same conditions for further 2 weeks, then transferred from the growth chamber to the laboratory and maintained under natural daylight at 19 and 28°C night-time and day-time temperatures.

Callus induction

Segments of cotyledonary and 1st foliage leaves were excised from 30 days-old seedlings. The segments were cultured in baby food jars containing MS medium supplemented with 30g L^{-1} sucrose, 8g L^{-1} agar and various combinations of (1 and 2mg L^{-1}) 2,4-D, (0.5, 1 and 2mg L^{-1}) IBA, (0.2, 0.4, 0.6, 0.8 and 1mg L^{-1}) BAP and 0.5mg L^{-1} KIN (Table 3). The jars were incubated in darkness at 25±2ºC. Callus was sub-cultured on the same medium after 3 weeks of culture. Fresh and dry weights of 6 weeks age calli were determined. The dry material was kept for assaying total flavonoid content.

Assay of total flavonoid content

Total flavonoids were extracted from callus of *R. pictus* by homogenizing 200mg of dry material in 20ml of 80% methanol. The homogenate was agitated on a rotary shaker at 100rpm and 25±2°C for 24h and then centrifuged at 6000rpm. Total flavonoid content was measured by the aluminum chloride colorimetric assay (Zhishen et al., 1999). An aliquot (1ml) of methanol extract or standard solution of quercetin (0.01-0.07mg ml^{-1}) was mixed with 4 ml distilled H_2O and 300µl of 5% $NaNO_2$. After 5min, 300µl of 10% $AlCl_3$ were added. At the 6th min, two ml of 1M NaOH solution were added and the total volume was made up to 10ml with distilled H_2O. The solution was mixed well, and the absorbance was measured against the reagent blank at 510nm. Total flavonoid content was expressed as mg quercetin equivalents g^{-1} dry weight.

Experiment design and statistical analysis

All experiments were of completely randomized design and repeated twice. Each treatment (media variants) consisted of 5 replicates per treatment, where each replicate consisted of a baby food jar containing three explants. Data presented here are means ±SE. One-way analysis of variance (ANOVA) was applied using SPSS V16 statistical program; the mean values were compared with Duncan's new multiple range tests at 5% significance level.

Results and Discussion

Shoot proliferation and multiplication

Both percentage of shoot induction and number of shoots per explant varied due to using different combinations of growth regulators. Control (Growth regulators-free) medium did not induce any shoots. Percentage of shoot induction on media with 2mg L^{-1} KIN + 0.5mg L^{-1} IBA, 2mg L^{-1} BAP and 2mg L^{-1} BAP + 0.2mg L^{-1} IBA reached 70-72%, while the highest percentage of shoot induction was achieved on media supplemented with 2mg L^{-1} TDZ (80%). BAP supplemented into MS medium mostly induced more shoots per explant in comparison to the other cytokinins, KIN and TDZ, (Table 1 and Fig. 1 A). The highest number of shoots per explant (12.6) was induced by 2mg L^{-1} BAP and it was associated with high percentage of shoot induction (70%). Our results support those found by Al Khateeb et al. (2017), who reported that BAP induced the best results in shoot multiplication from shoot apex of *R. cyprius* when compared with TDZ and KIN. In contrast, *Polygonum hydropiper* exhibited a preferential multiple shoot induction from shoot tip on KIN over BAP (Hasan & Sikdar, 2010). Effectiveness of BAP to induce multiple shoots was also reported on a variety of plants such as *R. vesicarius, Cassia alata* and *Rheum emodi* (Abo El-Soud et al., 2011; Ahmed et al., 2017 and Tabin et al., 2017). Superiority of BAP for shoot induction may be due to the ability of plant tissue to metabolize BAP more readily than other synthetic growth regulators (Kalia et al., 2007). However, increasing the level of BAP in the media to 3mg L^{-1} caused a significant decline in the number of shoots per explant, indicating that the need to optimize the concentrations of cytokinin was critical in shoot multiplication.

Addition of IBA to the media had variable effects on shoot formation from shoot tips of *R. pictus*. It affected the response of explants to the various cytokinins used in the current investigation. Adding 0.2mg L^{-1} IBA to 2mg L^{-1} KIN increased the number of shoots to nearly two-fold of that induced by 2mg L^{-1} KIN alone. Increasing the level of IBA to 0.5mg L^{-1} lowered that enhancement to one and a half. On the other hand, culturing

shoot tips on media supplemented with 2mg L^{-1} TDZ or 2mg L^{-1} BAP in combination with 0.2mg L^{-1} IBA decreased the percentage of shoot induction and the number of the induced shoots below those recorded with TDZ or BAP alone. The effect of addition of auxins to cytokinins in the regeneration media was diversely reported in the previous investigations. It was reported that inclusion of low concentration of auxins along with cytokinin enhanced shoot multiplication and induced higher number of shoots/explant as compared with cytokinin alone (Swain et al., 2016 and Ahmed et al., 2017). While, Kalia et al. (2007) found that adding NAA to BAP in the medium had a deleterious effect on shoot bud formation. In the present study, the obtained results indicated that the effect of IBA added to the micropropagation medium depends on the type and concentration of the cytokinin used in the growth regulators combination, suggesting that interaction between IBA and the utilized cytokinin exists.

TDZ is considered to be one of the most active PGRs for *in vitro* propagation system in a wide variety of plants (Guo et al., 2011). However, *R. pictus* shoots cultured on 2mg L^{-1} (Fig. 1 B) showed thick broad leaves that were wrinkled, curled and brittle all indicating symptoms of hyperhydricity (Dewir et al., 2014). The abnormal plant growth associated with high TDZ concentrations was demonstrated (Banerjee et al., 2004). Kadota & Nimii (2003) suggested that synthetic phenylurea derivatives (CPPU and TDZ) produce more hyperhydric shoots than with adenine derivatives (BAP and KIN).

In vitro root formation and acclimatization

It was reported that concentration of basal salts in medium has an important influence on root induction and quality (Shekhawat et al., 2015 and Kumari et al., 2016). *R. pictus* microshoots did not form roots when cultured on full strength MS media. While, half-strength MS media supplemented with NAA or IBA successfully induced roots (Table 2). Roots appeared after 3 weeks, and the ability of root formation was maximum (highest percentage of root induction; 40%, maximum number of roots; 3 and maximum root length; 3cm) with either 2mg L^{-1} NAA or 1mg L^{-1} IBA (Fig. 1 C). The promoting effects of reducing the salt level of medium on root induction were reviewed in several plant species (Dewir et al., 2016 and Kumari et al., 2016). Incorporation of growth regulators to the rooting media was necessary for root induction. However, IAA induced no roots from *R. pictus* shoots, even when applied with half-strength MS media (Table 2), indicating that root induction may be influenced also by the suitability of the exogenously added growth regulator. The superiority of IBA as effective auxin for inducing *in vitro* rooting over IAA and NAA was previously pointed out (Moharami et al., 2014; Swain et al., 2016 and Al Khateeb et al., 2017). IBA was reported to be the most successful auxin in root formation in several plant species (Thomas, 2007; Shekhawat et al., 2015 and Dewir et al., 2016), however an increase of IBA level (up to 2mg L^{-1}) decreased the percentage of root induction, root number and root length of *R. pictus* (Table 2). Similarly, Husain et al. (2007) observed that increased IBA concentration led to reduction of rooting ability of *Pterocarpus marsupium* along with callus formation.

TABLE 1. Effect of various combinations of growth regulators on shoot proliferation and multiplication of *Rumex pictus* using shoot apex after four weeks of culture.

Growth regulators (mg L^{-1})	Shoot induction (% of control)	No. of shoots/explant
2 KIN	60	3.60±0.33a
2 KIN+0.2 IBA	60	7.00±1.00bc
2 KIN+0.5 IBA	70	5.30±0.60ab
3 KIN	50	6.70±0.6abc
2 BAP	70	12.6±1.60d
2 BAP+0.2 IBA	72	8.80±1.10c
3 BAP	50	4.00±1.00ab
3 BAP+0.2 IBA	60	4.00±1.00ab
2 TDZ	80	5.40±0.51ab
2 TDZ+0.2 IBA	60	5.30±0.33ab
3 TDZ	60	8.60±0.88c

Values followed by the same letters are not significantly different ($P \leq 0.05$).

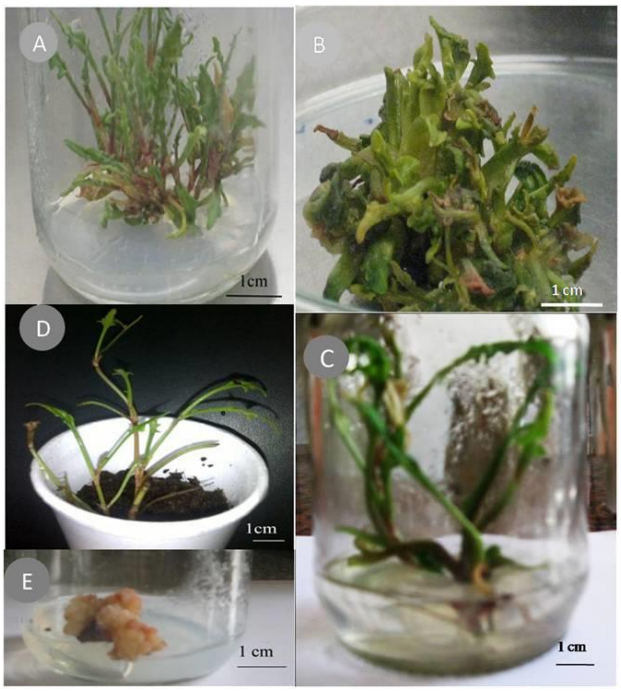

Fig. 1. Micropropagation and callus formation of *Rumex pictus*; A: Multiple shoots obtained from shoot tip explant cultured on MS medium supplemented with 2mg L^{-1} BAP for four weeks, B: Hyperhydricity observed on MS medium supplemented with 3mg L^{-1} TDZ, C: Roots induced on half-strength MS medium supplemented with 1mg L^{-1} IBA, D: Acclimatized plant and E: Three weeks old callus induced on MS media supplemented with 1mg L^{-1} 2,4-D and 0.4mg L^{-1} BAP using foliage leaf explant.

TABLE 2. Effect of NAA and IBA on *in vitro* root formation of *Rumex pictus* cultured for six weeks on half-strength MS.

Conc. of auxins (mg L^{-1})		Root induction (%)	No. of roots	Root length (cm)
NAA	IBA			
0	0	0	0	0
1	0	40	1	1.5
2	0	40	3	3
0	1	40	3	3
0	2	20	1	0.5

Plants with well developed roots were acclimatized by transferring to sterile peat moss soil, covered with transparent polyethylene bags, to maintain humidity, and kept in the growth chamber. During acclimatization, the plantlets were gradually transferred to regular environment by the help of irrigation with half-strength MS salts and gradual exposure to low humidity. Such acclimatized plantlets showed better survival when they were transferred to *ex vitro* conditions (Fig. 1 D). The plants scored 50% survival after one month of being in *ex vitro* conditions.

Callus induction and total flavonoid content

Callus was induced within 12-14 days of culture at the leaf margins and cut ends. The obtained calli were reddish yellow (yellow with red spots) in color, compact and globular in texture (Fig. 1 E). The results obtained from callus culture using cotyledonary leaf explants revealed that media with combination of 2,4-D and BAP were successful in callus induction, while those included IBA combined with either BAP or KIN did not show callogenesis (Table 3). In contrast, *R. vesicarius* cotyledonary explants exhibited better induction and proliferation of

callus on IBA than on 2,4-D (El-Shafey et al., 2016). Similar to our findings, MS media supplemented with 2,4-D and KIN were found to be suitable for callus induction and proliferation of buckwheat cotyledon tissue, while NAA in combination with KIN failed in inducing callus (Woo et al., 2000).

The percentage of callus induction, when cotyledonary leaves were used as explants, reached the highest value (88.8%) at combination of 1mg L^{-1} 2,4-D and 0.8mg L^{-1} BAP (Table 3). In addition, the highest callus frequency (100%) was recorded on combination of 1mg L^{-1} 2,4-D and 0.4mg L^{-1} BAP using foliage leaf explants (Table 3). However, in most cases, the highest callus frequency was not accompanied by the highest proliferation of callus. The various responses of callus frequency and proliferation suggest that during each stage (induction/proliferation) the plant cells have their different needs of auxins and cytokinins, and the level of auxins and cytokinins depends not only on their uptake from the extracellular source but also on their metabolism and endogenous interaction (Jiménez & Bangerth, 2001). The effective influence of combination of 2,4-D and BAP in enhancing callus frequency and producing maximum callus biomass was also observed on *Silybum marianum* and *Viola wittrockiana* (Cimino et al., 2006 and Wang & Bao, 2007).

TABLE 3. Effect of growth regulators on induction, fresh weight, dry weight and flavonoid content of 6 weeks age calli induced on cotyledon and foliage leaf explants of *Rumex pictus*.

Explant	Growth regulators (mg L^{-1})		Callus induction (%)	Fresh weight (g)	Dry weight (g)	Flavonoid content (mg Quercetin equivalent g^{-1} dry wt)
	2,4-D	BAP				
Cotyledonary leaf	1	0.2	66.6	0.56±0.05ab	0.035±0.004a	8.2±0.18e
	1	0.4	55.5	1.15±0.70b	0.079±0.039b	3.6±0.15a
	1	0.6	44.4	0.11±0.008a	0.013±0.001a	4.2±0.27ab
	1	0.8	88.8	0.20±0.020a	0.022±0.001a	3.7±0.15a
	1	1	22	0.04±0.016a	0.008±0.002a	8.4±0.30e
	2	0.2	25	0.06±0.029a	0.007±0.003a	5.5±0.4cd
	2	0.4	37.5	0.04±0.006a	0.005±0.001a	9.3±0.52f
	2	0.6	80	0.02±0.007a	0.003±0.001a	5.9±0.20d
	2	0.8	80	0.07±0003a	0.009±0.002a	4.9±0.09bc
Foliage leaf	1	0.2	92	0.45±0.038c	0.031±0.001a	6.1±0.28c
	1	0.4	100	1.09±0.024d	0.09±0.014b	5.3±0.16b
	1	0.6	75	0.31±0.023b	0.027±0.002a	4.7±0.04ab
	1	0.8	60	0.29±0.026b	0.038±0.002a	4.2±0.14a
	1	1	83	0.15±0.005a	0.014±0.002a	5.0±0.17b
	2	0.2	50	0.17±0.06a	0.017±0.007a	9.1±0.3d
	2	0.4	40	0.13±0.04a	0.016±0.003a	8.7±0.13d
	2	0.8	30	0.10±0.06a	0.010±0.006a	6.2±0.35c

Values followed by the same letters are not significantly different ($P \leq 0.05$)

The selection of proper combination of plant growth regulators was necessary to get high percentage of callus induction and high mass production. Previous studies found that callus, of other medicinal plants belong to the family Polygonaceae rather than *R. pictus*, grows better on media containing high cytokinin: auxin ratio (Liu et al., 2005; Wang et al., 2011 and Al Khateeb et al., 2017). On the other hand, the combination of 1mg L^{-1} 2,4-D and 0.4mg L^{-1} BAP was mostly the best combination for callus induction and proliferation, since it significantly induced the highest callus biomass (1.15 and 1.09g fresh wt and 0.079 and 0.09g of dry wt) from both cotyledonary and foliage leaves explants of *R. pictus*, respectively (Table 3). These differences may be due to the variation among genotypes and endogenous levels of hormones. It is worth mentioning that 1st foliage leaf explant was better than cotyledon in scoring the highest callus frequency and the highest dry mass production, emphasizing that selecting the appropriate explant source was important factor for successful callus induction and proliferation.

Flavonoids production and accumulation by plant tissue culture were reported for various medicinal plant species (Matkowski, 2008). Our results (Table 3) revealed that increasing the concentration of BAP above 0.2mg L^{-1} in combination with 1mg L^{-1} 2,4-D inhibited the accumulation of flavonoids in both types of cultures (cotyledonary and foliage leaves calli). Although 2mg L^{-1} 2,4-D induced lower callus growth with all combinations of BAP, it enhanced the accumulation of flavonoids (Table 3). The highest accumulation of flavonoids (9.3 and 9.1mg Quercetin equivalent g^{-1} dry wt) was recorded in callus culture (of cotyledonary and foliage leaves, respectively) supplemented with 2mg L^{-1} 2,4-D+ 0.4mg L^{-1} BAP and 2mg L^{-1} 2,4-D+ 0.2mg L^{-1} BAP. These results indicated that the accumulation of flavonoids in *R. pictus* callus necessitates the incorporation of higher concentration of the exogenously added auxin. Similarly, Masoumian et al. (2011) reported that auxins affected flavonoids production in callus culture of *Hydrocotyl bonariensis*, and supplementation of media with 2,4-D at 2mg L^{-1} gave the highest flavonoids production. Generally, it was noticed that most of the high records of flavonoid content were accompanied with lower callus induction and proliferation (Table 3), indicating that the growth of *R. pictus* calli and their production of flavonoids may behave antagonistically. Similar situation was found by Downey et al. (2013) and Chaabani et al. (2015), while working on callus of soybean (*Glycine max* L.) and *Crataegus azarolus* (hawthorn), respectively.

Conclusion

Our results suggested that BAP was the most suitable cytokinin for shoot multiplication on *R. pictus* shoot tip culture. It also contributed effectively in callus induction when added to 2,4-D containing media, particularly with the 1st foliage leaf that was the best explant for callus production and proliferation. In contrast to callus growth, accumulation of flavonoids was diminished by BAP but enhanced by increasing 2,4-D in culture medium. To the best of our knowledge this is the first study evaluating the effect of growth regulators on *in vitro* regeneration and callus induction of the endangered medicinal plant *R. pictus*.

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تأثير منظمات النمو على الإكثار الدقيق و استحثاث الكالوس ومحتوى فلافونويدات الكالوس من نبات الحمصيص .*Rumex pictus* Forssk نبات طبى مهدّد بالانقراض

نادية محمد الشافعي، مني سيد، إيناس شعبان أحمد، علا حمودة، صلاح الدين علي خضرى
قسم النبات و الميكروبيولوجى – كلية العلوم – جامعة بنى سويف – بنى سويف – مصر.

تتناول الدراسة الحالية تأثير منظمات النمو على التكاثر الدقيق و استحثاث الكالوس ومحتوى فلافونويدات الكالوس من نبات الحمصيص (*Rumex pictus* Forssk) الطبى المهدّد بالانقراض. حيث تم تقييم توليفات مختلفة من الكينتين (KIN) و بنزيل أمينو بيورين (BAP) و الثايديازورون (TDZ) و اندول حمض البيوتيرك (IBA) من أجل الإكثار الدقيق من القمة النامية للمجموع الخضرى للنبات. ولقد اتضح أن الوسط الغذائى موراشيج و سكوج (MS) محتويا على 2 مليجرام/لتر من بنزيل أمينو بيورين هو الأفضل للإكثار الدقيق (نسبة انتاج المجاميع الخضرية هى 70% و 12.6 مجموع خضرى مستحدث لكل جزء نباتى مستخدم /shoot explant). نتجت أعلى نسبة لاستحداث الجذور عند استخدام نصف تركيز الوسط الغذائى (1/2 MS) مضافا إليه 2 مليجرام/لتر من نفثالين حمض الخليك (NAA) أو 1 مليجرام/لتر من اندول حمض البيوتيرك. أحدث 1 مليجرام/لتر من 2 و4- ثنائى كلوروفينوكسي حمض الخليك (2,4-D) مضافا الى 0.4 مليجرام/لتر من بنزيل أمينو بيورين أعلى انتاج للكالوس من الأجزاء النباتية المستخدمة، ولا سيما الورقة الخضرية الأولى التي اتضح أنها أفضل جزء نباتى يمكن استخدامه لزراعة و نمو الكالوس. وعلى النقيض من نمو الكالوس، فإن مركبات الفلافونويدات قد تضاءل تراكمها فى الكالوس عند استخدام بنزيل أمينو بيورين ولكنه تحسن بزيادة ثنائى كلوروفينوكسي حمض الخليك في وسط الزراعة. و يمثل الإكثار الدقيق واستحثاث الكالوس أحد التقنيات السريعة لإكثار هذا النبات الطبى الهام والحفاظ عليه من خطر الأنقراض بالإضافة إلى إنتاج المركبات الثانوية الهامة منه معمليا حيث يتم تناول كل هذا و لأول مرة بالدراسة الحالية.