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Morphogenic responses *via* "organogenesis" of Salix calli in relation to the produced salicin *in-vivo* and *in-vitro.*

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ABSTRACT

This study was carried out aiming to study the morphology and growth behavior of produced calli of *Salix safsaf* Forssk plant and estimates their morphogenic capacities *via* organogenesis "indirect regeneration". Moreover, investigate the active ingredient Salicin content (g/100gDW) produced in shoot either *in vivo* or/and *in vitro* in both developed calli and regenerated shoots. The most common outcomes proved that; survivals percentages varied between 0 to 99 % as both Clorox concentration and soaking period changed. Immersion explants in 30% Clorox solution containing 3-5 drops of Tween 20 for 20min and followed by three times rinses in sterile distilled water showed no contaminated explants. The optimum concentration of auxin (NAA) was 0.25 mg/l in combination with different concentrations of cytokine (BA) produced the most heaviest calli dry weight. The suitable concentrations of BA for mass callus production from stem, leaf and buds explants were 0.25, 0.5 and 1 mg/l, respectively. Leaf explant produced the heaviest average calli fresh weight 0.48 g/jar as compared with the other explants shoot 0.31 g and bud 0.35 g. Relative to the control either NAA or BA treatments caused significant increase in calli fresh weight. The highest percentage of regeneration capacity (83%), maximum number of shoots (5 shoots) and the highest shoot length (3.82 cm) were recorded with culturing of stem calli on MS medium augmented with 0.25 mg/l BA. The highest recorded value of root formation percentage 85.2% was recorded with MS medium supplemented with IBA 0.2mg/l. The average computed Salicin content in salix shoots grown *in vivo* was 4.37 g/100g DW as compared with 1.52 g/100gDW produced in shoots aged 5 weeks formed *in vitro*. However, the estimated content of salicin in developed callus was 0.07 g/100gDW.

Keywords: *Salix safsaf* Forssk, indirect regeneration, Salicin.

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INTRODUCTION

Willows, sallows, and osiers from the genus Salix, around 400 species, of deciduous trees and shrubs, found primarily on containing moist soils in cold and temperate regions of the Northern Hemisphere. Most species are known as willow, but some narrow-leaved shrub species are called osier, and some broader-leaved species are referred to as sallow (from Old English selah, related to the Latin word salix, willow). Some willows (particularly arctic and alpine species) are low-growing or creeping shrubs; for example, the dwarf willow (*Salix herbacea*) rarely exceeds 6 cm (2 in) in height, though it spreads widely across the ground. That identified the antibacterial and antifungal activities as well as the antiviral, antiparasitic, and other pharmacologically active substance activities in higher plants. The extracts of certain plants are known to yield active antimicrobial substances which have been documented as phytochemicals of the genus of the family, and reported as the toxicity of the plants. Many components that are derived from medical and dietary plants possess potential chemopreventive properties [1]. In addition, willow species are widely used for phytoremediation of polluted soils [2,3] because of their wide-spreading root systems and fast growth. Willows can also survive and grow in hydroponic systems in the presence of heavy metals at above normal critical concentrations [4] .

Tissue culture is the technique of maintaining plant tissue in an artificial medium *in vitro* under control condition. Propagation of plants through tissue culture has become an important and popular technique. The continuous supply of sterile plantlets will overcome the contamination problem and reduce the time for sterilization process. However, many factors contribute to the ability of a specific tissue to form callus such as medium and plant growth regulators. Moreover, it is believed that adventitious shoot regeneration from callus may causes somaclonal variation [5] while direct shoot regeneration from leaf or stem explants may eliminate such undesirable variations [6].

In vitro propagation from adventitious or axillary buds is a useful technique for producing clonal plantlets. There are several reports of axillary shoot multiplication in willow species [7-12] . However, because the shoots arise from preformed buds, there are only two published reports on *in vitro* regeneration of *Salix* species from adventitious buds or somatic embryos [13-14] and only were able to regenerate significant numbers of plantlets [14] . Callus culture was initiated from leaf explants of mature *Salix exigua* (Nutt.), [13] induced somatic embryos from callus derived from pistils of *Salix viminalis* (L.), cultured first on medium with 2,4-D and BAP and then on regeneration medium with different combinations of plant growth regulators (PGRs).Our objective was to develop a protocol for *in vitro* callus induction and regeneration of Salix plants. It was aimed to study the growth behavior of produced calli and estimates their morphogenic capacities *via* organogenesis "indirect regeneration" along with investigate the regeneration capacity of different types of explants. Moreover, to investigate the active ingredient Salicin content (g/100g DW) produced in shoot either *in vivo* or/and *in vitro* in both callus and regenerated shoot.

MATERIALS AND METHODS

This study was carried out in the Center Tissue Culture Laboratory, Agricultural Botany Dept. Faculty of Agriculture, Cairo University, during the period from 2012 to 2014. Plant material was secured from the uppermost branches of willow trees (*Salix safsaf* Forssk) grown in Orman Botanic Garden, Giza governorate, Egypt. Explants include buds, shoot tips and leaf were excised from the chosen twigs. Explants were subjected to different sterilization treatments using sodium hypochlorite. NaOCl (Clorox 5%) at five concentrations 20, 25, 30, 35, 40 and 45% for 10, 15, 20 and 25 min. with ethanol alcohol 70% for 5 seconds before. Data were recorded for 5 weeks to calculate survival percentages.

Explants were cultured on MS medium supplemented with different combinations of NAA and BA (0.25,0.50 and 1.00 mg/l), 3% sucrose, 0.2 % gel right and, the medium pH was adjusted to 5.7 – 5.8. The culture medium was sterilized by autoclaving at 120 ºC for 20 min. Average callus fresh weight was estimated from the resultants calli of the three types of explants (leaf, stem and buds) after five weeks (each sample consisted of mass of callus grown on one explants). Obtained calli from shoot tip explants were subcultured on MS medium which supplemented with (0.00 mg/l NAA + 0.25 mg/l BA), (0.00 mg/l NAA + 0.50 mg/l BA), (0.25 mg/l NAA + 0.25 mg/l BA) and (0.50 mg/l NAA + 0.50 mg/l BA). The data following were recorded after 5 weeks of cultivation.

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Shoots regeneration capacity was calculated according the following formula:

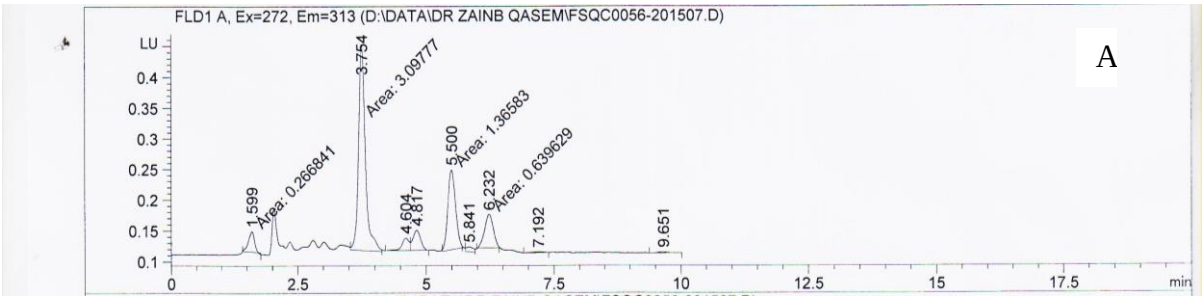
$$\text{Shoots regeneration capacity} = \frac{No.of\ explant\ formed\ shoots}{total\ no.of\ explants} \times 100$$

Number of shoots/calli inoculums : Average number of shootlets.

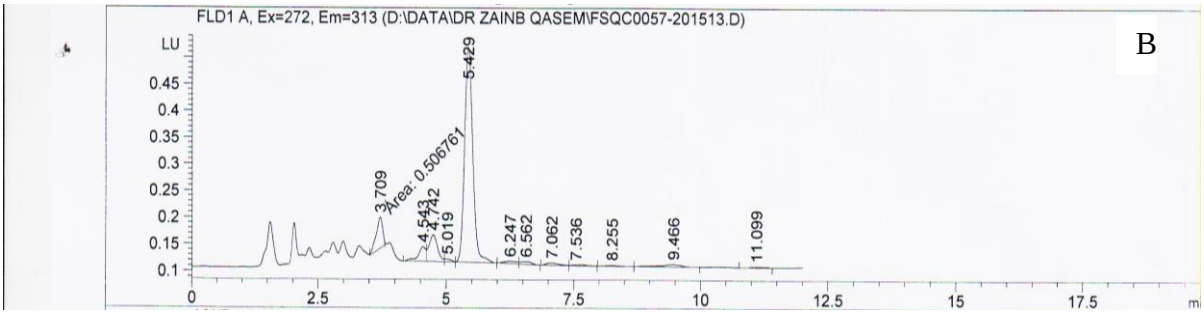
Roots formation was studied from indirect regenerated shoots (1-2 cm) derived from calli cultures of stem explants which showed the highest shoot regeneration capacity, maximum number of shoots, highest shoot length were re-cultured onto ½ MS medium salts strength supplemented with 0.2 mg/l of the following auxins; NAA, IBA and IAA. Data were recorded after 5 weeks to estimate percentage of rooted shoots, average number of roots per shoot and average root length (cm).

The active ingredient salicylic derivatives produced in Salix shoots *in vivo* as well as those produced *in vitro* in both developed calli and regenerated shoots at 5 weeks old was detected by HPLC analysis described by [15]Ancatiu *et al*. (2012).

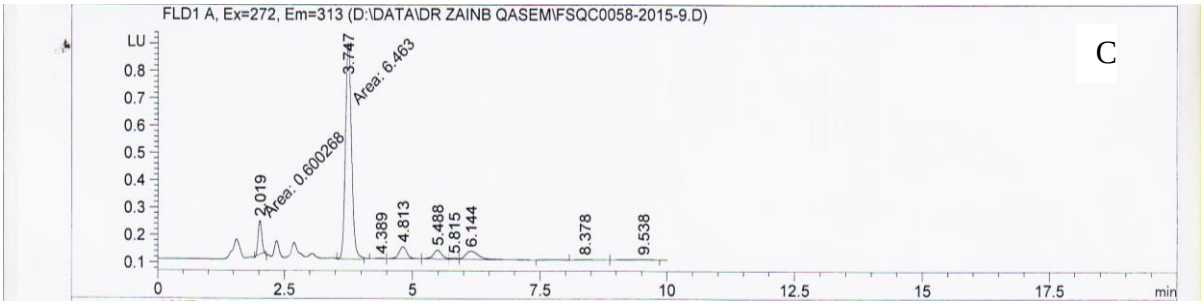
Data were subjected to the convenient statistical analysis modules described by [16]Snedecor and Cochran (1980) and means were separated using least significant differences LSD test at 5% level of certainty.



A: HPLC of salicin in shoot (*in vivo*) of *Salix safsaf* Forssk (3.752)



B: HPLC of salicin in shoot (*in vitro*) of *Salix safsaf* Forssk (3.709)



C: HPLC of salicin in calli of *Salix safsaf* Forssk (3.247)

Plate (1): HPLC sheets (A,B &C) of salicylates derivatives produced in *Salix safsaf* Forssk shoots *in vivo* and those produced *in vitro* in regenerated shoots and developed calli 5 nderson old.

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RESULTS AND DISSCUION

Sterilization

Previous reports indicated difficulties in establishing *in vitro* vegetative propagation of Salix due to high contamination indexes observed in applied cultures. Data presented in Table (1) show effect of different concentrations of Clorox (NaOCl) and soaking period on survival percentage of cultured explants of *Salix* plants. The survivals percentages varied between 0 to 99 % as both Clorox concentration and soaking period changed. It is obvious that immersion explants in 30% Clorox solution containing 3-5 drops of Tween 20 for 20 min and followed by three times rinses in sterile distilled water this treatment showed no contaminated explants. So, the average number of contaminated plants decreased with the increase of both the hypochlorite concentration and the soaking period. Different survival percentages were observed by application of different Clorox concentrations as well different soaking periods, Table (1). Similar outcomes were reported by many workers with numerous plant species among them [17-18].

Table 1: Effect of different concentrations of Clorox and soaking periods on explants survival percentages of *Salix safsaf* Forssk.

Clorox concentrations	Time (min) B				
	10	15	20	25	Mean (A)
20	0.00	0.00	0.28	0.39	0.17
25	0.14	0.38	0.40	0,59	0.31
30	0.44	0.49	0.99	0.18	0.53
40	0.52	0.78	0.00	0.00	0.33
Mean (B)	0.28	0.41	0.42	0.19	0.32
LSD**5%** (A) 0.16 (B) 0. 11 (AXB) 0.21					

Calli fresh weight (g)

Callus proliferation was observed within 3 weeks following culture initiation Fig. (1). The three types of explants (leaf, stem and buds) were used for estimating calli fresh weights. Initially, white to greenish, spongy calli appeared, but it later turned to hard yellow or watery brown callus. Callus formation was observed in all explants types as well with all of the PGR treatments.

Callus formation was significantly affected by the three studied factors; the used explants, the applied PGR and the adopted concentration. Statistically, these factors showed significant effect on the average obtained callus weight (g). Moreover, significant effect was determined due to the interaction between these factors. Therefore, regardless the effect of the other factors under study, the optimal combination that produced the highest callus weight, will be pronounced out in this study.

Data tabulated in Table (2) clearly shows that the effect of supplementation MS medium with different combinations of NAA and BA on achievement of calli production from buds, leaf and stem explants of *Salix safsaf* Forssk. The highest value of fresh weights 0.47, 0.60 and 0.68 (g/jar) were recorded with stem, bud and leaf explants, respectively. The optimum concentration of auxin (NAA) was 0.25 mg/l in combination with different concentrations of cytokine (BA). The suitable concentrations of BA for mass callus production from stem, leaf and buds explants were 0.25, 0.5 and 1 mg/l, respectively. The favorite explants for calli production were leaf (Fig.1-A), buds (Fig. 1-B) and stem (Fig.1-C) explants, respectively.

With regard to the used explants type, it is obvious that the used explants types significantly affected the resulted callus weight. Generally, regardless the applied medium, leaf explant produced the heaviest average callus fresh weight 0.48 g as compared with the other explants shoot 0.31 g and bud 0.35 g, Table (2). The chosen explant was determined according to the study performed by [19]Satu-lyyra *et al* (2006).

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Data in Table (2) show that regardless the explant types, stem, leaf and bud, the average recorded callus fresh weight for 0 NAA treatments was 0.351 g. While, the corresponded recorded fresh weight in case of 0 BA was 0.313 g. The most convenient PGR combination for producing the heaviest fresh callus 0.481 g. was 0.25 (NAA)+ 0.50 (BA). The other applied combinations produced intermediate callus fresh weight values. These outcomes are in accordance with those previously reported by [20-23]Tejavathi *et al.* (2001), Dedicova *et al.* (2000), Shiv-Ratan *et al.* (2001) and Blinstrubiene *et al.* (2004).

Table 2: Effect of different combinations of NAA and BA on calli fresh weight (g/jar) induced from bud, leaf and stem explants of *Salix safsaf* Forssk.

MS medium supplemented with:		Average calli fresh weight (g/jar)			
		Explants			Mean (B)
NAA	BA	Stem	Bud	Leaf	
0	0	0.25	0	0.35	0.20
	0.25	0.36	0.22	0.58	0.39
	0.5	0.31	0.25	0.62	0.39
	1	0.21	0.27	0.34	0.27
Average		0.28	0.18	0.47	0.31
0.25	0	0.4	0.35	0.52	0.42
	0.25	0.47	0.4	0.53	0.47
	0.5	0.44	0.52	0.68	0.55
	1	0.2	0.60	0.49	0.43
Average		0.37	0.46	0.55	0.47
0.5	0	0.3	0.32	0.34	0.32
	0.25	0.45	0.4	0.49	0.45
	0.5	0.2	0.5	0.51	0.40
	1	0.43	0.42	0.5	0.45
Average		0.345	0.41	0.46	0.41
1	0	0.3	0.25	0.36	0.30
	0.25	0.31	0.31	0.43	0.35
	0.5	0.25	0.51	0.52	0.43
	1	0.23	0.42	0.42	0.36
Average		0.27	0.37	0.43	0.36
Mean (A)		0.32	0.36	0.48	
LSD 5%					
(A)		0.03	0.03	0.04	
(B)		0.02	0.01	0.01	
(AxB)		0.04	0.03	0.02	

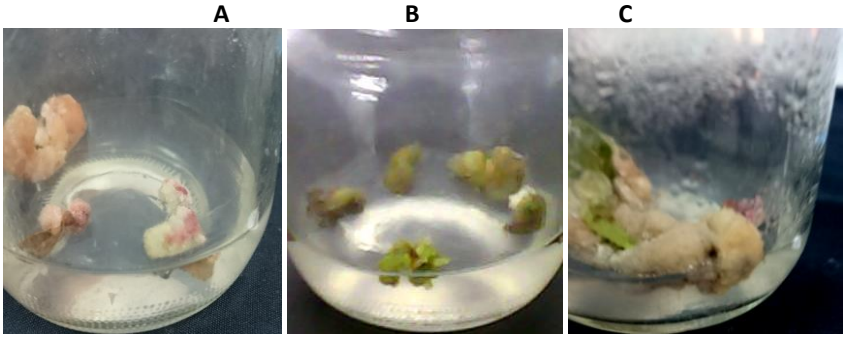

Figure 1: Calli production from leaf (A), bud (B) and stem (C) explants of *Salix safsaf* Forssk cultured on MS medium supplemented with 0.25 mg/l NAA in combinations with different concentrations of BA(0.50 mg/l,1.00 mg/l and 0.25 mg/l). Cultures were incubated under light condition 16/8h at 26 ± 2° C

The interaction between the used explants and the applied growth regulator was significant. Relative to the control either NAA or BA treatments caused significant increase in callus fresh weight. Since, no callus was formed in case of bud explants combined with (0 NAA + 0 BA). Whereas the same treatment showed 0.25 and 0.35 g fresh callus with stem and leaf explants respectively, table 2. Moreover, the heaviest fresh callus

weight 0.68 g. was obtained with leaf explants treated with (0.25 mg NAA + 0.50 mg BA). The obtained results are in harmony with those previously reported by many researchers among them [20].

Shoot regeneration capacity (%)

After transferring the formed calli to shoot regeneration media, purple or green spots appeared on their surface in some treatments which subsequently converted to shoot primordia and then to shoots with several leaves (Fig. 2).

For enhancement of indirect shoots regeneration an equal inoculums ~ 250 mg/ of stem calli were subcultured on each jar containing MS medium fortified with different concentrations of NAA and BA. The percentage of indirect shoot regeneration, number of regenerated shoots and shoot length (cm) were recorded after 5 weeks of incubation under light condition 16/8h. The highest percentage of regeneration capacity (83%), maximum number of shoots (5 shoots) and the highest shoot length (3.82 cm) were recorded with culturing of stem calli on MS medium augmented with 0.25 mg/l BA (Table 3 and Fig. 2). However, the lowest regeneration capacity (50 %), minimum number of regenerated shoots (2) and lowest shoot length (2.87 cm) were recorded with supplementation of MS medium with an equal concentration (0.5 mg/l) from NAA and BA.

Shoot regeneration in the medium containing 0. 25 mg/l BA was better than the medium containing 0.5 mg/l BA. A sample of regenerated shoots in the fifth week of callus culture in 0.25 mg/l BA has been shown as the best shoot regenerated results. Callus proliferation had predominate response on the media containing 0.5 mg/l NAA or in combination with 0.5 mg/l BA. Formed calli in these treatments were green and friable but regenerate shoots with very low frequencies 50%. Regenerated shoots in these treatments were longer than those in the other treatments and had shoot length ranged between 2.87 to 3.83 cm. It was also noticed that, the applied PGR concentration resulted in variation in number of shoots/ explants. Since the medium containing 0.25 mg/l BA produced an average of 5 shoots per explant. However, the medium containing 0.5 mg/l NAA plus 0.5 mg/l BA produced an average of 2 shoots per explant. These results of shoot regeneration from callus have been reported by many investigators among them [24] achieved suitable shoot formation in *Portulaca grandiflora* by transferring the hypocotyl-derived callus to MS medium without plant growth regulator, whereas we found that the presence of cytokinin is necessary for shoot regeneration from leaf-derived calli, since the calli which were cultured in MS medium without plant growth regulator failed in regenerating shoots. Moreover, we found that in high concentrations (10 or 15 µM) of cytokinins, addition of NAA promotes shoot regeneration from callus, which it corresponds with the results of Rossi- [25]. It seems that high concentrations of BA are necessary to shoot regeneration in *Mose Rose*. Also. it can be deduced that it in higher levels of BAP (10 or 15 µM) shoot regeneration is promoted by adding of NAA, while in lower concentration of BA (5 µM) it helps the callus proliferation. Some researchers have reported that an appropriate combination of NAA and BA stimulated shoot formation [26-28. Our findings confirm their results.

Table 3: Effect of supplementation of MS medium with different concentrations of NAA and BA on indirect Salix shoots regeneration induced from stem calli.

NAA(mg/l)	BA(mg/l)	Reg.(%)	NO. Shoot/calli inoculums	Shoot length. (cm)
0.00	**0.00**	**0**	**0**	**0**
0.00	**0.25**	83	5	3.82
0.00	**0.50**	66	4	3.38
0.25	**0.25**	58	3	3.03
0.50	**0.50**	50	2	2.87
LSD5%		**2.4**	**6.66**	**0.16**

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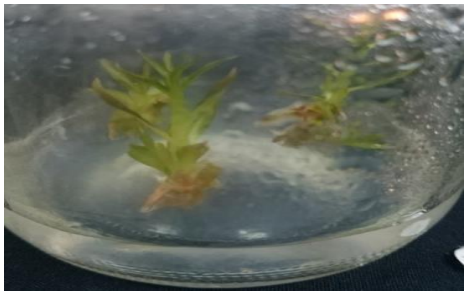

Figure 2:. Indirect shoot regeneration from stem calli of *salix safsaf* Forssk cultured on MS medium supplemented with 0.25 mg/l BA and incubated under light condition 16/8h for 5 weeks at 26 ± 2 ºC.

Root formation

Table 4: Effect of supplementation of MS medium with different concentrations of IBA, NAA and IAA on indirect Salix root formation percentages, average number of roots/explant and average root length (cm) induced from stem calli.

MS supplemented with	% Root Formation	Number of roots	Root length (cm)
Zero (PGR)	0	0	0
IBA (0.2 mg/l)	85.2	5.0	2.32
NAA (0.2mg/l)	70.2	3.0	2.88
IAA(0.2mg/l)	63.3	2.0	3.82
LSD5%	0.37	0.2	0.17

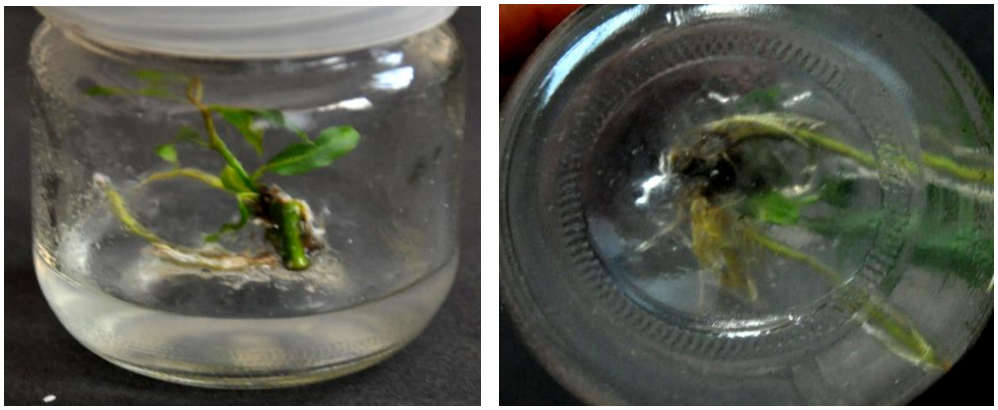

Figure 3: Root formation on shoots of *salix safsaf* Forssk cultured on 1/2 MS medium supplemented with IBA (0.2 mg/l)

Root initiation was carried out by using stem segment as source of explant. The explants showed swelling within 12-14 days of inoculation; however shoots formation started after 20-25 days at the margins of the explant and subsequently spread over the entire segment. In the present study, the earliest and highest percentage root induction and growth of roots were observed on MS medium supplemented with IBA 0.2 mg/l and/or IAA 0.2mg/l. The explants failed to produce callus on MS medium without growth regulators. Data presented in Table (4) reveal that the highest recorded value of root formation percentage 85.2% was recorded with MS medium supplemented with IBA (0.2mg/l), while the lowest percentage was 63.3% recorded with MS medium supplemented with IAA (0.2mg/l) counted after 5 weeks. The highest value for number of roots 5 per explant was recorded with MS medium supplemented with IBA (0.2mg/l), nevertheless the lowest number was 2 per explant recorded with MS medium supplemented with IAA (0.2mg/l). However, the longest root 3.82cm was recorded with MS medium supplemented with IAA (0.2mg/l), while the shortest root was 2.88 cm recorded with MS medium supplemented with NAA (0.2mg/l). Similar finding was observed by [30]Reeta *et al*(2008). The maximum seed germination, callusing and regeneration in callus were obtained in control seeds. In the present study, the maximum root initiation from callus was observed on MS medium supplemented with IBA 5 mg/l. The earlier workers [5,30] developed roots from shoots obtained via callus.

Similar findings were observed by [30]Reeta *et al*(2008). In the present study, the maximum root initiation from callus was observed on MS medium supplemented with IBA 5 mg/l. The earlier workers [5,30] developed roots from shoots obtained *via* callus. These results were in close agreement with those of [29]Aswath and Choudhary (2002) they found combination of IBA and IAA or using each one alone were more effective for roots formation than either NAA or IAA alone.

Salicylates content (g/100g DW)

The active ingredient salicin produced in Salix shoots *in vivo* was quntitively determined as well as those produced *in vitro* in both developed calli and regenerated shoots at 5 weeks old. The average computed salicin content in salix shoots grown *in vivo* was 4.37 g/100g dry weight as compared with 1.52 g/100gDW produced in shoots formed *in vitro*. However, the estimated content of salicin in developed callus was 0.07 g/100g DW Plate (1). It was reported that the most abundant phenolics in Salix were Salicylates (Salicin, 29-*O*-acetylsalicortin, acetylsalicortin and saligenin). The highest content of total salicylates was found in the young shoot tips: over 10% (w/w) on a dry weight basis. The mature leaves contained about 6% (w/w) salicylates and the stems under 3% (w/w) on a dry weight basis. This results were relatively in harmony with those reported by [31]Teija and Maija-Riitta (2000) who mentioned that, the relative amount of salicin was the same in all plant parts (12%–14%), but the amount of 29-*O*-acetylsalicortin varied according to the part: in the stems the proportion of acetylsalicortin was only 8%, but in the leaves it was as high as 24%, and in the shoot tips 17%. The levels of saligenin and diglucoside of salicin were low in whole shoot (0.7%–2.2%).

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