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RESEARCH ARTICLE

Effects of basic media and plant growth regulators on Direct-somatic embryogenesis induction in vegetative strains obtained from the tissue culture of digitalis purpurea

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ABSTRACT

This study aimed to investigate the effects of certain nutrient medium types enhanced with plant-growth regulators on the direct induction of somatic embryogenesis in vegetative strains produced and propagated from a tissue culture of *Digitalis purpurea*. The results show that MS medium was superior to other tested nutrient media-B5, LS, and 5C01—in terms of the days required to initiate direct somatic embryogenesis induction and induction rate. In contrast, the LS medium resulted in the highest number of embryos. Moreover, 2 mg/L BA (benzyl adenine) was superior to similar concentrations of BAP (benzyl aminopurine) and Kin (kinetin) in the MS medium, whereas BAP performed better in LS and B5. On the other hand, auxin 2.4D (dichlorophenoxy acetic acid) inhibited somatic embryogenesis, as did NAA (naphthalene acetic acid) and IAA (indole acetic acid) when added individually. Regarding the type of explant, the stems showed the induction rates and numbers of direct-somatic embryos produced in all the basic media.

Keywords: *Digitalis purpurea*; Vegetative strains; Direct-somatic embryogenesis; Explant; Basic media

INTRODUCTION

Despite attempts to achieve food security and economic growth, biotic and abiotic constraints, such as diseases, pests, weeds, drought conditions, and other production constraints (Barceloux, 2009; Bull et al., 2011), limit the production that is necessary to meet the growing demands for the plants, food, and medicinal crop supplies. The changing climatic conditions also pose challenges that must be addressed (Chaudhary et al., 2015).

Conventional farming techniques have various limitations, such as low fertility, unsynchronized production, and limited genetic knowledge (Nassar and Ortiz, 2010); for instance, producing improved plant genotypes using conventional breeding programs is a long process that takes many years (Ceballos et al., 2004; Rudi et al., 2010).

Somatic embryogenesis is one of the most common in vitro regeneration methods to achieve the rapid and accurate propagation of many plant species (Chaudhary and Dantu, 2019). Moreover, in vitro regeneration has been employed as an approach to restore and resettle endangered species through plant genetic improvement (Adsul et al., 2019; Dabul et al., 2009); additionally, tissue culture techniques provide reliable and alternative methods for breaking the dormancy phase and overcoming the obstacles to germination in many plant species (Chang et al., 2020). It has been proposed that plant-growth regulators, such as auxins and cytokinins, provide a new mechanism of embryonic response due to their active participation in the regulation of the cell division cycle (Chen et al., 2016; Francis and Sorrell, 2001). The plant species, explant types, nutrient medium, and incubation conditions, such as light and temperature, have been shown to significantly influence the success rate and efficiency of regeneration (Chen et al., 2019).

Somatic embryogenesis occurs directly when embryos are developed from plant tissues or indirectly when embryos or

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shoots are produced after passing through the callus stage (Gutirrez-Mora et al., 2012; Jain and Gupta, 2005; Lema-Rumińska et al., 2013; Mujib and Samaj, 2006). Added plant-growth regulators play an important role in somatic embryogenesis (Chen et al., 2016). The balance of the growth regulators added to the culture medium is the main factor for the differentiation of direct somatic embryos into organs (Shirin and Rana, 2007). However, other factors, such as the plant species and culture conditions, play a role in the success of in vitro direct somatic embryogenesis. Chen et al. (2019); Haliloglu and Aydin (2016) confirmed that the explant quality, nutrient medium, plant-growth regulators, and incubation conditions are important factors that directly affect the efficiency and success of plant vegetative propagation through the direct organogenesis of somatic embryos.

Digitalis purpurea is one of the most important medicinal plants in the Plantaginaceae family (Olmstead, 2002), as it is a natural source of cardiac glycosides, such as digoxin, which is utilised in the treatment of congestive heart failure. In 1990, the US Food and Drug Administration (FDA) approved using digoxin. Because of its influence on the sodium-potassium pump (Chen et al., 2015). Moreover, several studies have been recently published on the effectiveness of cancer treatment (Platz et al., 2011; Stenkvist et al., 1982).

The conventional cultivation methods for the Digitalis genus are inadequate and risky, as they have a low seed germination rate and are ineffective in producing an adequate stock of plant matter (Verma et al., 2016b), in addition to the damage caused by the random collection of plants. Despite attempts to produce varieties of *D. purpurea* and *D. Lanata* that contain high levels of cardiac glycosides through conventional cultivation methods, the plants' production of the active substances has not been stable, and the work needed to improve it requires long-term, cumbersome, and expensive breeding programs (Verma et al., 2016b).

Therefore, in recent decades, biotechnology research has focused on in vitro methods for the propagation and improvement of digitalis, especially *Digitalis purpurea*, through the direct organogenesis of direct-somatic embryogenesis, to realize a rapid multiplication of the plant material (Bhusare et al., 2018).

In the previous studies of Bhusare et al. (2020); Lioshina and Bulko, (2014); Pérez-Alonso et al. (2018), they stimulated direct somatic embryogenesis of *Digitalis spp*, and the results varied according to the nutrient medium and plant growth regulators concentration.

This research focuses on the effective establishing protocols for induction and growing direct somatic embryogenesis for *Digitalis purpurea* explants based on the type of basic medium improved with plant-growth regulators.

EXPERMINTAL PROCEDURE

Location of research

This investigation was accomplished at the Lab of the Biotechnology of Medicinal-Plants "National Commission for Biotechnology, Damascus, Syria", as well as at the Tissue Culture Laboratories "Department of Plant Biology, Faculty of Science, Damascus University, Syria", during the period of 2018–2021.

Plant material

Four vegetative strains (DPM1, DPM2, DPM3, and DPM4) were obtained from the micropropagation of *D.purpurea* in the National Commission for Biotechnology laboratories.

Direct somatic embryogenesis induction

Three types of explants (leaves, stems, and roots) were cultured on the nutrient media MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), LS (Linsmaier and Skoog, 1965), and 5C01(Vollosovich et al., 1979) in the presence of a series of frequented concentrations (0.25, 0.5, 1, and 2) mg/L of cytokinines (Kin, BAP, and BA) and auxins (NAA, 2.4-D and IAA), single and overlapping. For each treatment, 20 explants were cultured with 4 replicates under highly sterile conditions in the laminar (JSCR-1200 SB). The explants were stored at 25 ± 2 °C for 70 days and with 8–16 h dark-light cycles. The following preliminary observations were recorded: the days required to initiate direct somatic embryogenesis induction in each medium, the days required for buds to differentiate into plants, the induction rate for each tested nutrient medium and each used explant, and the number of shoots resulting from each explant.

Statistical analysis

All the tests were accomplished utilised by split-split-plot (SSP) designs, with using of 4 replications. The data were analyzed after tabulation using Mstat-C software in order to estimate the values of the least-significant differences (LSD) at the significance of 0.01%, and the coefficient of variance (CV%).

RESULTS AND DISCUSSION

Required days to initiate the direct somatic embryogenesis induction

The sample results of Table 1 demonstrated the advantage of the MS-medium, with significant differences. In terms

Table 1: Days required to initiate direct somatic embryogenesis induction

	MS				B5				LS				5C01			
	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean
Control	9	9	16	11.3[b]	14	14	17	15[a]	15	13	17	15[a]	17	16	24	19[a]
DPM1	8	9	15	10.7[a]	14	16	19	16.3[b]	16	16	19	17[b]	16	18	23	19[a]
DPM2	9	10	15	11.3[b]	15	17	19	17[c]	15	16	20	17[b]	17	18	25	20[b]
DPM3	10	10	16	12[c]	15	18	20	17.7[d]	16	17	21	18[c]	19	20	25	21.3[c]
DPM4	10	10	17	12.3[c]	16	18	21	18.3[e]	16	18	21	18.3[c]	19	19	26	21.3[c]
Mean	9.2[a]	9.6[b]	15.8[c]		14.8[a]	16.6[b]	19.2[c]		15.6[a]	16[a]	19.6[b]		17.6[a]	18.2[b]	24.6[c]	

The different letters in the table indicate to the significant differences

of the number of days needed to initiate somatic embryo induction, with an average of 9.2 days when using the leaves as an explant, 9.6 days when using the stems, and 15.8 days when using the roots, compared with other tested media (B5, LS, and 5C01). Moreover, only the two vegetative strains, DPM1 and DPM2, performed the best in the MS medium, as 10.7 and 11.3 days, respectively, were needed to start inducing the embryoids, with significant differences from the other used vegetative strains and the control, whereas the control was superior in B5 and LS, with an average of 15 days for each. As for the effect of the explant, the results show that the leaves were significantly exclusive, as the leaves needed the lowest number of days to initiate somatic embryo induction, followed by the stems and, finally, the roots; this could be explained by the abundance of young meristematic tissues in the leaves and their potential energy, making them able to divide more than in the stems and roots (Ichihashi and Tsukaya, 2015). The superiority of the MS medium may be attributed to its richness in certain nutrients, especially the major elements such as magnesium and calcium; the importance of these elements in vital reactions in the plant; and the balance of the concentrations of the elements of the medium with the contents of the plant extracts of the nutrients without toxic effects; this, in turn, leads to the ideal concentrations for the growth, development, and exposure of the cultivated plant part (Mineo, 1990).

The advantage of the MS medium was due to the presence of certain nutrients, especially macro-elements such as magnesium and calcium. Magnesium activates certain enzymes, such as AMP pyrophosphorylase, hexokinase, and glucokinase. It affects the synthesis of proteins and the formation of chromosomes, helps to increase the absorption and movement of phosphorous within plant cells, and plays a role in building ATP molecules and nucleic acids (Mahler, 2004), and its concentration increases in the meristematic tissues. Calcium has a positive effect through its role in regulating amino acid metabolism and its effect on enzyme functions. Consequently, an appropriate balance of the concentrations of these elements with the explant content of nutrients leads to the growth, development, and exposure of the cultivated plant part (Mineo, 1990). The experimental results agreed with the published results by Verma et al. (2016a), who reported that the MS-medium was superior compared with the LS, B5, and CHE for direct somatic embryo induction in the plant parts of *Crocus* sp. The findings of this experimental work agreed with the published results by Chen et al. (2014). They confirmed the difference in the induction periods according to the medium type, its composition, and the explant extract type used in tissue culture when studied on *Glossogyne tenuifolia*. Also, the results converge with the findings of Bhusare et al. (2018) for *Digitalis lanata* regarding the required days to initiate direct somatic embryo induction on an MS medium.

Days required for somatic embryos to differentiate into vegetative buds

The subsection refers to the number of days during which the induction responses lead to differentiation into plants or buds that can be cultivated. The results in Table 2 reveal that the MS medium required the lowest average number of days for somatic embryos to start forming, with an average of 40.2 days when using the leaves as an explant, 36.9 days when using stems, and 48.2 days when using the roots, compared with the other tested media (B5, LS, and 5C01). The DPM1 vegetative strain performed the best, as it needed 40.7 days in the MS medium to start forming somatic embryos. It significantly differed from the control and other vegetative strains used in all the tested nutrient media. As for the effect of the explant type, the results show that the stems were significantly superior to the leaves and roots in all the tested nutrient media, as they needed the lowest number of days for the differentiation of somatic embryos into cultivable buds to start; this is attributed to the rate of nutrient accumulation in the stems and the consistency of their plant hormone content with the amount of the medium, in addition to the potential energy in the cells forming the buds and their ability to divide, which stimulated their growth and development (Ghimire et al., 2016). On the other hand, the superiority of the MS medium may be attributed to its richness in certain nutrients, especially macro-elements, such as magnesium and calcium; the importance of these elements in vital reactions in the plant; and the balance of the concentrations

of the medium's elements with the explant content of the nutrients not having a toxic effect; this, in turn, leads to the ideal concentrations for the growth, development, and exposure of the cultivated plant part (Mineo, 1990).

These findings are similar to the published results by Bhusare et al. (2018) for *D.lanata* in terms of the days required for the display and differentiation of somatic embryos in the MS medium; the published results by Verma et al. (2016a) from their study on *Crocus species* comparing the MS medium and LS, B5, and CHE media; and the results of Shen et al. (2018) for *Tolumnia louise*.

Effect of the explant type on the rate of direct somatic embryo induction

The findings of experimental work in Table 3 show the advantage of stems in terms of the rate of direct-somatic embryo induction as compared with the leaves and roots, as the rate was 73% in the MS medium, 63% in the B5 medium, 54% in the LS medium, and 23% in the 5C01 medium. This superiority could be explained by the high meristematic activity in the buds present in them, in addition to the difference in the tissue type that constitutes the explant and its potential energy, the difference in the number of cells capable of dividing (Bhusare et al., 2018), and the role and level of endogenous plant hormones and additive growth regulators, which greatly affects the responses of plant parts to the processes of growth and development (Mastuti et al., 2017).

The lowest induction rate for somatic embryos was found when using roots as the explant, which may be due to the anatomical structures of the root and its internal content of plant-growth regulators.

As for the type of medium, the MS medium resulted in the highest response rate for somatic bud induction compared to other media, regardless of the explant type used. However, the results show that the 5C01 medium was not suitable for direct somatic embryogenesis induction. Furthermore, the control was superior, exhibiting the highest induction rate when using stems (67.5%) and roots (16.25%), followed by the DPM1 vegetative strain, which showed the highest induction rate when using leaves as explants (26.5%) compared to the rest of the vegetative strains.

These results are consistent with what was indicated by Bhusare et al. (2018) when studying *Digitalis lanata* and confirmed the superiority of the stems over explants in the MS medium, where the stems responded better than the leaves, and this may be due to the meristematic activity in the buds on the stems, which appeared clearly compared to that in the leaves, in which the response was limited to the edges of the leaf ends (Ghimire et al., 2016). This was confirmed by Moon et al. (2013) in a study on the effect of the explant type on the somatic embryogenesis induction of *Oplopanax elatus*, where different responses could be achieved from different explants of the same plant. Moreover, these findings agreed with the published findings by Verma et al. (2016a) regarding the superiority of the MS-medium in terms of the rate of the direct-somatic embryo growth response in *Crocus species*.

Effect of the explant type on the average number of direct-somatic embryos

The findings of experimental work in Table 4 show the superiority of the stems, compared to the leaves and roots, in terms of the number of formed direct-somatic embryos,

Table 2: Days required to form direct somatic embryos

	MS				B5				LS				5C01			
	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean
Contro	41	38	49	42.7^{c}	43	41	51	45^{c}	43	39	52	44.7^{e}	46	45	56	49^{d}
DPM1	39	36	47	40.7^{a}	41	38	48	42.3^{a}	40	36	49	41.7^{a}	44	43	51	46^{a}
DPM2	39	36	48	42^{b}	40	39	48	42.3^{a}	40	37	51	42.7^{b}	45	44	52	47^{b}
DPM3	41	37	48	42^{b}	43	40	49	44^{b}	42	39	50	43.7^{c}	45	43	52	46.7^{b}
DPM4	41	37	49	42.3^{b}	44	41	50	45^{c}	42	39	51	44^{d}	46	44	54	48^{c}
Mean	40.2^{b}	36.8^{a}	48.2^{c}		42.2^{b}	39.8^{a}	49.2^{c}		41.4^{b}	38^{a}	50.6^{c}		45.2^{b}	43.8^{a}	53^{c}	

The different letters in the table indicate to the significant differences

Table 3: The effect of explant type on the rate of direct somatic embryogenesis induction

	Leaves					Stems					Roots				
	MS	B5	LS	5C01	Mean	MS	B5	LS	5C01	Mean	MS	B5	LS	5C01	Mean
D.P (Co)	30	35	15	10	22.5^{b}	85	75	80	30	67.5^{a}	25	15	20	5	16.25^{a}
DPM1	35	35	20	15	26.25^{a}	80	70	80	25	63.75^{b}	20	15	15	5	13.75^{b}
DPM2	25	25	20	10	20^{c}	75	65	70	20	57.5^{c}	15	10	15	0	10^{c}
DPM3	20	15	10	5	12.5 ^{e}	65	60	65	25	53.75^{d}	10	10	10	0	7.5^{d}
DPM4	20	20	15	10	16.25^{d}	60	45	55	15	43.75 ^{e}	10	5	10	5	7.5^{d}
Mean	26^{a}	26^{a}	16^{b}	10^{c}		73^{a}	63^{c}	70^{b}	23^{d}		16^{a}	11^{c}	14^{b}	3^{d}	

The different letters in the table indicate to the significant differences

Table 4: The average number of direct-somatic embryos resulting from the studied explants

	Leaves					Stems					Roots				
	MS	B5	LS	5C01	Mean	MS	B5	LS	5C01	Mean	MS	B5	LS	5C01	Mean
D.P	7.3	6.8	7.4	4.1	6.4[a]	9.6	8.7	8.9	5.2	8.1[a]	3.1	2.5	2.9	1.7	2.55[a]
DPM1	6.9	5.3	7.6	3.5	5.83[b]	9.4	8.9	9.2	4.9	8.1[a]	2.6	2.1	3.4	1.6	2.43[a]
DPM2	6.5	5.1	7.3	2.8	5.43[c]	8.3	7.8	8.7	5.1	7.48[b]	2.1	1.9	3.1	1.3	2.18[b]
DPM3	5.8	5.2	6.9	2.5	5.1[d]	8.4	7.3	8.6	4.3	7.15[c]	1.8	1.7	2.9	1.1	1.88[c]
DPM4	5.6	4.9	6.7	2.3	4.88[e]	6.5	6.9	7.6	4.4	6.35[d]	1.9	1.6	2.7	1.2	1.85[c]
Mean	6.42[b]	5.46[c]	7.18[a]	3.04[d]		8.44[b]	7.92[c]	8.6[a]	4.78[d]		2.3[b]	1.96[c]	3[a]	1.38[d]	

The different letters in the table indicate to the significant differences

as they produced 8.44 embryos in the MS, 7.92 embryos in B5, 8.6 embryos in LS, and 4.78 embryos in 5C01, and this could be explained by the high meristem activity in the buds, in addition to their potential energy. The lowest number of formed somatic embryos was found when the roots were used as explants, which may be due to the anatomical structures of the root and its internal content of plant-growth regulators. As for the medium type, the LS medium retained the maximum number of formed direct-somatic embryos compared to other media according to the type of used explant, followed by the MS medium, while 5C01 provided the lowest number of somatic embryos. The control was superior, producing the highest number of formed direct-somatic embryos, followed by the DPM1 vegetative strain, without significant differences between the stems and roots compared to the rest of the used vegetative strains.

The results of this study agree with Gurel et al. (2011) on the species *Digitalis davisiana Heywood*, in terms of the significant differences in the induced number of direct-somatic embryos relying on the plant species, type of explant, and components of the nutrient media. They are also similar with the results of Adsul et al. (2019) in terms of the production of direct somatic embryos in the MS medium from the stems of *Ceropegia mohanramii* and *C. noorjahnia* (Chavan et al., 2014); the findings of Verma et al. (2016a) study with MS medium, which showed decent results regarding the average number of somatic embryos according to the type of explant for *Crocus species* compared to those obtained in LS, B5, and CHE media; and the published findings of Chen et al. (2014) study on the average number of shoots resulting from the propagation of *Glossogyne tenuifolia*.

Effect of the type and concentration of single-state cytokinin on direct somatic embryogenesis induction

The results recorded in Table 5 and Fig. 1 illustrate that the concentration of 2 mg/L for all the tested cytokinins (Kin, BAP, and BA) was better than the other studied concentrations for the different types of medium and types of used vegetative strain. The highest induction rate in the MS medium, 58% on average, was observed with 2 mg/L Kin; the control was superior to the other vegetative strains in this medium, with statistical significance. However, 2 mg/L BAP yielded the maximum induction rate for direct-somatic embryos in the B5 medium, with an average of 69%, with the DPM1 vegetative strain found to be superior, with significant differences. In general, the highest induction rate was found when using BA at a concentration of 2 mg/L in the MS medium and with different concentrations of the studied regulators, with an average of 76%; the control was found to be the best in the four-nutrient media, followed by the DPM1 vegetative strain. Additionally, the results show that, when using high concentrations, 1 to 2 mg/L, of cytokinins, BA was generally superior to BAP and Kin in the direct induction of direct-somatic embryogenesis in most of the nutrient media. At low concentrations of cytokinins, the response in terms of direct-somatic embryo induction differed according to the cytokinin type. These findings are similar to the results of Shen et al. (2018), confirming that Kin resulted in relatively little response in terms of somatic embryo induction from *Tolumina louise* explants, while the results of Huang et al. (2014) showed that BA was the most effective at inducing the largest number of shoots when planting *Gentiana Scabra* explants in the MS medium, and this confirmed the effectiveness of BA and its inducing role for the uptake of nutrients and growth regulators by cells, thus achieving a higher growth rate compared to BAP and Kin (Mok and Mok, 1994). These results are consistent with Mongomake et al. (2015), showing that BAP remarkably induced the organogenesis induction response in *Manihot esculenta Crantz*. Consequently, this indicates the preferences of explants of certain plant species for certain nutrient media and certain cytokinins, which is reflected in the acidity of the medium and the compatibility of its nutrient content with the internal content of the explant and, thus, in the response rate for direct somatic embryo induction. This may be due to the fact that plants with the ability to accumulate high levels of biologically active cytokinins are more amenable and preferable than the in vitro methods used in the induction, formation, and regeneration of plants (Hill and Schaller, 2013). Moreover, several studies have confirmed that cytokinins are effective in direct somatic embryogenesis

Table 5: Effects of type and concentration of single-state cytokinins on direct somatic embryogenesis induction

		KIN mg/l					BAP mg/l					BA mg/l				
		0.25	0.5	1.5	2	Mean	0.25	0.5	1.5	2	Mean	0.25	0.5	1.5	2	Mean
MS	D.P	30	55	70	75	57.5^{a}	35	50	70	80	58.75^{a}	25	45	65	85	55^{a}
	DPM1	35	45	60	70	52.5^{b}	25	30	60	80	48.75^{b}	25	40	70	85	55^{a}
	DPM2	30	30	40	55	38.75^{c}	20	20	45	55	35^{c}	15	35	65	75	47.5^{b}
	DPM3	25	30	40	45	35^{d}	15	25	40	60	35^{c}	15	35	55	70	35.75^{c}
	DPM4	20	25	35	45	31.25^{e}	15	20	40	55	32.5^{d}	20	20	40	65	36.25^{c}
	Mean	28^{d}	37^{c}	49^{b}	58^{a}		22^{d}	29^{c}	51^{b}	66^{a}		20^{d}	35^{c}	59^{b}	76^{a}	
B5	D.P	30	45	55	65	48.75^{a}	25	40	45	80	47.5^{a}	25	50	65	75	53.75^{b}
	DPM1	25	25	35	55	35^{b}	30	35	40	85	47.5^{a}	35	45	70	75	56.25^{a}
	DPM2	10	15	45	45	28.75^{c}	25	30	40	75	42.5^{b}	35	50	60	65	52.5^{b}
	DPM3	10	10	20	30	17.5^{d}	25	30	35	65	38.75^{c}	25	50	55	60	47.5^{c}
	DPM4	5	10	10	25	12.5^{e}	20	25	35	40	30^{d}	15	45	45	55	40^{d}
	Mean	16^{d}	21^{c}	33^{b}	44^{a}		25^{d}	32^{c}	39^{b}	69^{a}		27^{d}	48^{c}	59^{b}	66^{a}	
LS	D.P	20	35	45	65	41.25^{a}	35	40	45	70	47.50^{b}	30	40	55	65	47.5^{a}
	DPM1	20	25	40	60	36.25^{b}	30	45	50	75	50^{a}	25	35	45	60	41.25^{c}
	DPM2	15	30	35	50	32.5^{c}	25	35	45	65	42.5^{c}	25	40	50	60	43.75^{b}
	DPM3	20	25	45	55	36.25^{b}	20	40	40	65	41.25^{c}	20	30	45	55	37.5^{d}
	DPM4	15	25	35	50	31.25^{c}	15	30	35	50	32.5^{d}	20	25	35	50	32.5^{e}
	Mean	18^{d}	28^{c}	40^{b}	56^{a}		25^{d}	38^{c}	43^{b}	65^{a}		24^{d}	34^{c}	46^{b}	58^{a}	
5C01	D.P	10	15	20	20	16.25^{a}	15	15	20	20	17.5^{a}	15	20	25	25	21.25^{a}
	DPM1	10	10	15	15	12.5^{c}	10	15	15	15	13.75^{b}	10	15	20	20	16.25^{b}
	DPM2	5	10	10	15	10^{e}	10	10	10	15	11.25^{c}	10	10	15	20	13.75^{c}
	DPM3	5	15	10	15	11.25^{d}	5	10	15	15	11.25^{c}	15	15	15	20	16.25^{b}
	DPM4	10	15	15	20	15^{b}	5	10	10	10	8.75^{d}	10	10	15	15	12.5^{d}
	Mean	8^{d}	13^{c}	14^{b}	17^{a}		9^{d}	12^{c}	14^{b}	15^{a}		12^{d}	14^{c}	18^{b}	20^{a}	

The different letters in the table indicate to the significant differences

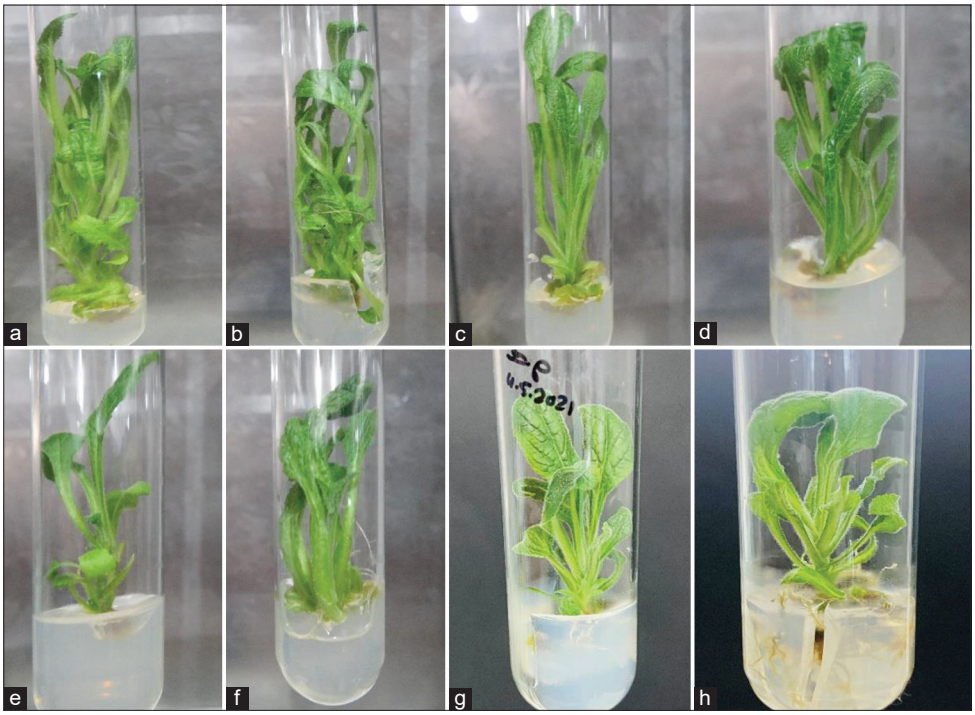

Fig 1. The effect of cytokinins on the induction rate for direct somatic embryos. (a)control (MS + 2 mg/L BA); (b)DPM1 strain (MS + 2 mg/L BA); (c)DPM1 strain (B5 + 2 mg/L BAP); (d)DPM1 strain (LS + 2 mg/L BAP); (e)control DP (5C01 + 2 mg/L BA); (f)DP (MS+2mg/L BAP); (g)DPM1 strain (MS+2 mg/L); (h)DP(B5+2 mg/L BAP)

induction from different explants, especially leaves (Hong et al., 2008).

As for the medium type, the MS-medium, in general, resulted in the maximum response rate for direct-somatic

embryo induction, for the different types of cytokinin, except in the case of using low concentrations of BAP and BA, for which the induction rates were more significant in the B5 and LS media, while the 5C01 medium resulted in the lowest induction rate for all the studied treatments, confirming its inappropriateness for achieving the goal of this study. The control was outperformed in terms of the induction rate by most of the nutrient media, regardless of the type of cytokinin used.

The results also show the formation of minor cases of callus on cutting surfaces in the explants of some treatments, as shown in Fig. 1c,d, and this was due to the compatibility of the added cytokinin concentration with the internal concentrations of auxins, which were at quantities that do not impede the targeted growth and are not sufficient to induce the roots or the brown callus that hinders growth. This is what is expressed in the state of the total balance between auxin and cytokinin, resulting from the total concentrations of the added growth regulators and endogenous plant-growth hormones (Mastuti et al., 2017).

Effect of the concentration and type of single-state auxin on direct-somatic embryogenesis induction

The addition of auxins in their single states stimulated the formation of callus-like cellular aggregates on the edges and surfaces in contact with the nutrient medium, and this was an obstacle to direct regeneration because the nutrients and potential energy stores in the plant tissues were distributed between the formation of the callus and the formation of somatic embryos, which led to stunting, a darkening of the tissues of the formed callus, and the death of many parts of the plants that were unable to continue to survive (Fig. 2(a-h)).

The results also show an increase in the browning rate of the ends contacting the surface of the medium with an increase in auxin concentration, and the levels of browning and stunting were 85% at 1 and 2 mg/L concentrations of 2.4D compared to NAA and IAA in the MS and LS media, while the lowest response was recorded in the B5 medium, in addition to the high rate of tissue browning of the explant when other auxins were added in other tested media. The high rates of browning reflected the significant decrease in direct somatic embryogenesis from the explant, so treatment with single auxins was not suitable for the direct induction of somatic embryos. This is similar to the published results by Shen et al. (2018) study on the *Tolimian Louise* species, in which it was confirmed that 2.4D alone retarded the induction of somatic embryos to a high degree, and 92–100% of the explants showed a state of darkening and dying in the formed callus cells or in the

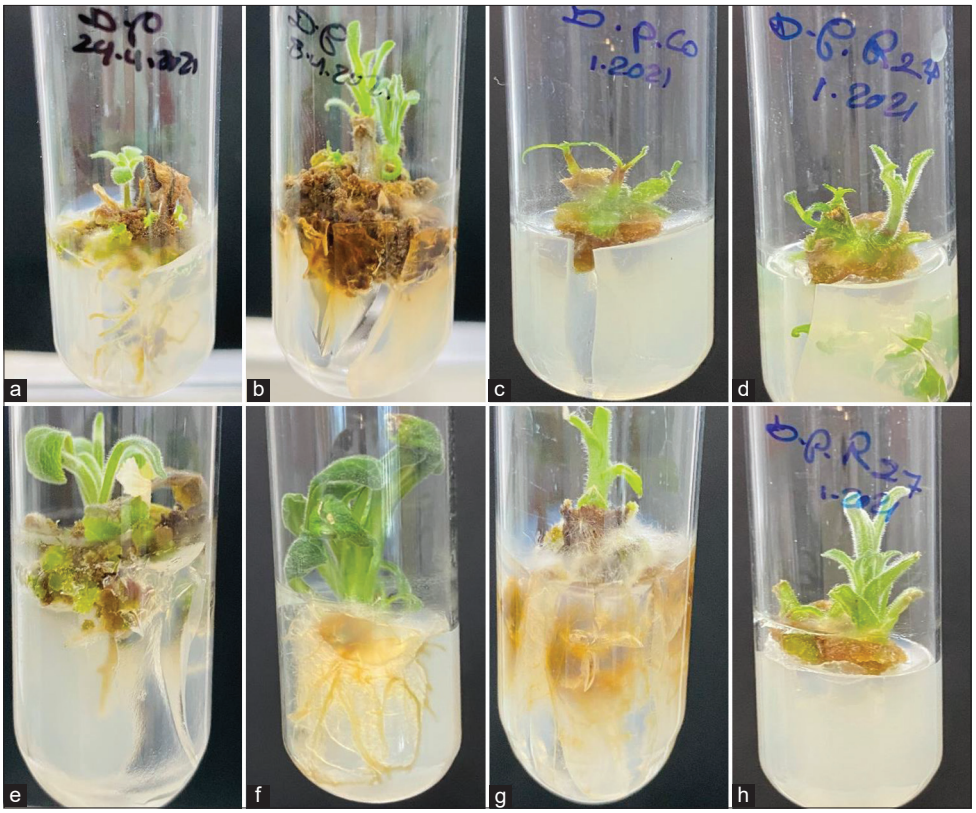

Fig 2. Effect of auxin concentrations on the formation of direct somatic embryos. (a)control (MS + 2 mg 2.4D); (b)control (B5 + 2 mg 2.4D); (c)control (LS + 2 mg NAA); (d)DPM1 strain (MS + 2 mg NAA); (e)DPM1 strain (LS + 2 mg 2.4D); (f)DPM1 strain (MS + 2 mg IAA); (g)DPM3 strain (MS + 2 mg IAA); (h)strain DPM4 (MS + 2 mg NAA).

explant itself. The findings of this research also agreed with the results of Chen and Chang (2001) study on the species *Oncidium*, the results of (Chung et al., 2007, 2005) study on the species *Dendrobium*, and the results of Kuo et al. (2005) and Gow et al. (2009) studies on *Phalaenopsis*, all of which confirmed that 2.4D resulted in significant inhibition of the direct somatic embryo development of the studied species.

The results of the study also show that higher concentrations of IAA resulted in a tendency for explants, in all the nutrient media, towards the formation of roots (Fig. 2 f), which differed in their length and number according to the media type, vegetative strains, and used explant type. The leaves in LS and B5 outperformed those in the other media in terms of directing explants towards root formation, and the strength of the roots was enhanced when roots were used as explants in the MS medium supplemented with IAA (Fig. 2 f,g). These results are consistent with what was confirmed by Gurel et al. (2011) in their study on the species *D.davisiana Heywood* and about the tendency of IAA to stimulate rooting in somatic cultures, and similar to the findings of Adsul et al. (2019) regarding the role of IAA in the root formation of direct-somatic embryos in a study on *Ceropegia mohanramii*. Ma and Xu (2002) indicated that NAA was not capable of inducing or leading to the formation of somatic embryos from the meristematic leaves of the cassava plant. Consequently, tables showing the results for the effect of adding single-state auxins on the induction of direct-somatic embryos of certain vegetative strains of *D. purpuria* are excluded.

Effect of the co-interference (concordances) of auxins and cytokinins on direct induction of somatic embryogenesis

High concentrations of auxins (2.4D, NAA, and IAA) at concentrations of 1 and 2 mg/L added to low-grade concentrations (0.25 and 0.5 mg/L) of the cytokinins Kin, BAP, and BA directed the tissues of the explant towards the formation of calluses or brownish swellings that hindered the direct formation of somatic embryos, whereas equal concentrations of auxins and cytokinins maintained the state of undifferentiated cells (calluses) at the cutting surfaces and at the edges. This is consistent with the results of Adsul et al. (2019), showing that higher concentrations of auxin led to the formation of calluses at the cut ends of the micro buds, while there was an effect of increasing the number of buds from the combinations enhanced with low concentrations of auxins (0.25 and 0.5 mg/L), which is reliable with the results of Fatima et al. (2009) study on *Digitalis lanata Ehrh*. Therefore, all the results regarding the hormonal combinations of the high concentrations of auxins and low concentrations of cytokinins were excluded, as were their balanced concentrations, which directed the formation of calluses or prevented the development of direct somatic embryos, while the treatments that included low concentrations of auxins with high concentrations of cytokinins were maintained.

Effect of the kinetin-with-auxin interaction on the rate of induction direct-somatic embryogenesis

The results in Table 6 and Fig. 3 show that the hormonal compatibility combination (2 mg/L Kin + 0.25 mg/L NAA) produced the highest induction rate of 70% in the MS-medium, higher than the rates in the different studied media, with superiority found for the control. The hormonal compatibility combination (2 mg/L Kin + 0.5 mg/L 2.4D) in the B5 medium resulted in a 65% induction rate, and that in the LS medium, 60%; these were better than the rates for the other concentrations, other treatments, and all the studied vegetative strains, including the control. However, the induction coefficients for the control and the vegetative strains in the 5C01 medium retained the lowest values in terms of the induction rate for direct-somatic embryos compared to the different studied media.

The superiority of NAA over both 2.4D and IAA when interacting with Kin, in terms of the induction ratio, is explained by the greater ability of the cells to absorb NAA and, possibly, NAA's longer intracellular activity compared to the other auxins (Hartmann et al., 2002), especially IAA, which was characterized by its ability to bind to cytoplasm components or its conversion to other hormonally inactive analogues, and its rapid decomposition and enzymatic catabolism.

By comparing the results of Tables 6 and 7, it can be observed that the addition of auxins at low concentrations resulted in a significant and clear difference in the treatments with the LS and B5 media, whereby the low amount of auxins enhanced the rate of direct somatic embryo induction in the studied treatments.

These results converge with the findings of Bhusare et al. (2018) study on *D.lanata* in terms of the response to somatic embryo induction in the presence of Kin in the MS medium. However, our results are contradicted by Fatima et al. (2009) on *Digitalis lanata Ehrh*, which confirmed a lack of preferability for the use of kinetin in direct somatic embryo induction due to the superiority of other cytokinins such as BA.

The effect of the benzylaminopurine (bap)-with-auxin interaction on the rate of direct-somatic embryogenesis induction

The findings of this reseach work in Table 7 and Fig. 4 demonstrate that the hormonal compatibility combination

Table 6: The effect of the kinetin-with-auxin interaction on the rate of direct somatic embryogenesis

		KIN (1 mg/L)						KIN (2 mg/) L					
		2.4D		NAA		IAA		2.4D		NAA		IAA	
		0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5
MS	D.P	75	70	70	60	45	60	75	75	75	65	55	65
	DPM1	70	60	65	55	40	45	70	65	75	65	50	65
	DPM2	65	45	60	50	35	40	65	65	70	65	50	65
	DPM3	60	40	50	40	35	40	60	65	65	55	45	60
	DPM4	60	40	50	35	30	40	55	60	65	50	50	60
	Mean	66	51	59	48	37	45	65	66	70	60	50	63
B5	D.P	55	65	35	45	50	55	55	75	35	50	55	55
	DPM1	50	60	30	35	45	45	55	70	30	45	45	50
	DPM2	55	50	30	40	40	45	50	65	25	40	45	45
	DPM3	50	45	25	35	35	45	50	60	25	35	40	45
	DPM4	45	45	25	30	35	50	50	55	20	35	40	40
	Mean	51	53	25	37	41	48	52	65	27	41	45	47
LS	D.P	55	55	50	60	35	45	65	70	55	60	50	55
	DPM1	50	55	45	55	35	40	60	65	50	60	45	50
	DPM2	35	45	40	50	30	40	60	60	45	55	35	50
	DPM3	45	50	45	45	30	35	55	60	45	55	35	45
	DPM4	35	35	30	40	25	30	35	45	40	45	30	35
	Mean	44	48	42	50	31	38	55	60	47	55	39	47
5C01	D.P	30	35	35	35	25	30	35	40	30	35	25	30
	DPM1	25	30	20	25	25	25	30	35	35	40	25	30
	DPM2	20	25	20	20	20	25	30	35	30	35	20	25
	DPM3	15	25	15	20	15	25	20	30	35	40	20	20
	DPM4	15	20	10	15	15	20	20	25	25	30	15	20
	Mean	21	27	20	23	20	25	27	33	31	36	21	25

Table 7: The effect of the BAP-with-auxin interaction on the induction rate for somatic embryos

		BAP (1 mg/L)						BAP (2 mg/L)					
		2.4D		NAA		IAA		2.4D		NAA		IAA	
		0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5
MS	D.P	65	60	65	70	45	50	70	65	70	80	55	55
	DPM1	65	55	65	70	40	50	70	70	75	80	50	60
	DPM2	55	50	60	65	45	45	65	60	65	75	45	55
	DPM3	50	50	60	60	40	45	60	55	65	70	50	60
	DPM4	45	45	55	55	35	40	50	45	50	65	45	50
	Mean	56	52	61	64	41	46	63	59	65	74	49	50
B5	D.P	60	70	65	70	45	50	60	75	75	75	50	55
	DPM1	55	70	65	75	45	45	65	75	75	80	45	55
	DPM2	50	60	55	70	45	45	60	65	65	75	40	40
	DPM3	50	55	60	70	40	45	55	60	50	65	40	45
	DPM4	45	60	55	60	35	40	45	65	60	70	35	45
	Mean	52	63	60	69	42	45	57	68	65	73	42	48
LS	D.P	60	65	55	55	45	50	60	60	50	55	45	45
	DPM1	55	65	45	50	35	45	50	55	45	45	35	40
	DPM2	50	60	50	55	35	40	45	55	40	45	30	35
	DPM3	45	60	45	50	35	35	45	50	35	40	35	35
	DPM4	40	55	35	50	30	30	40	50	30	35	25	30
	Mean	50	61	46	42	36	40	48	54	40	44	34	37
5C01	D.P	45	55	45	50	30	35	45	50	40	45	30	40
	DPM1	30	40	30	35	25	30	30	35	35	40	25	35
	DPM2	25	30	25	25	15	20	20	30	30	30	25	30
	DPM3	25	30	20	25	15	15	20	25	25	30	20	25
	DPM4	25	25	20	20	10	15	20	20	25	25	20	20
	Mean	30	36	28	31	19	23	27	32	31	34	24	30

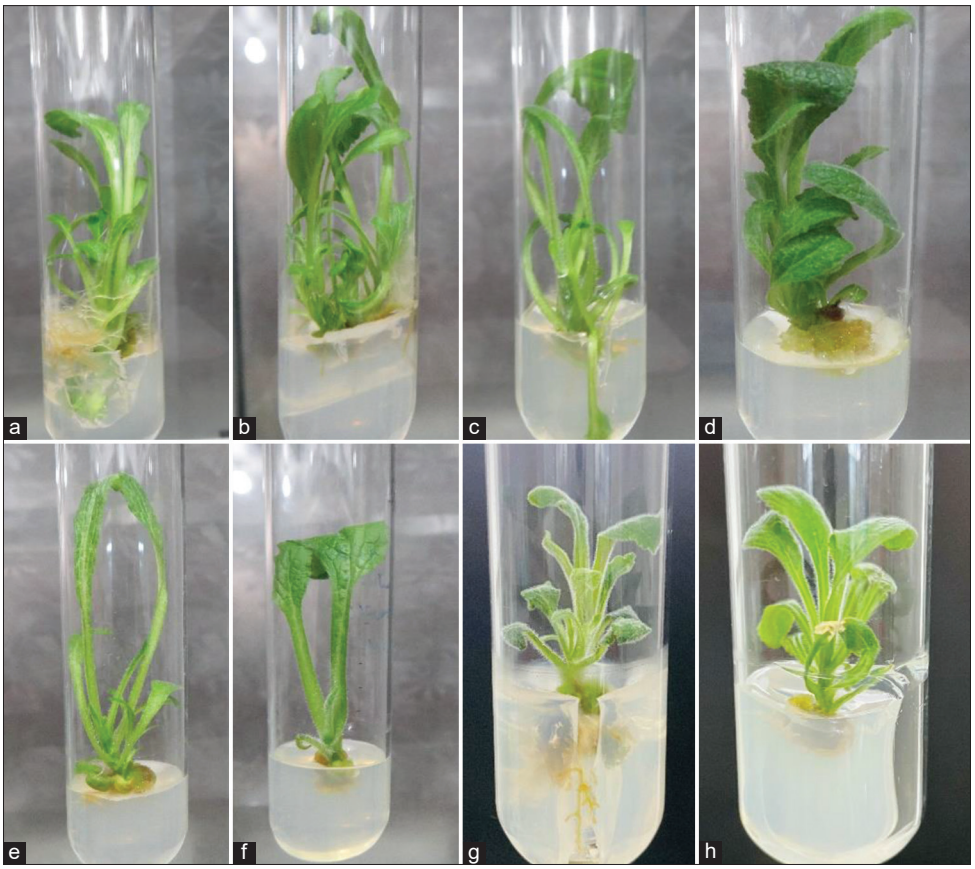

Fig 3. The effect of the kinetin-with-auxin interaction on the direct induction rate for somatic embryos. (a)control (MS + 0.25 mg NAA + 2 mg Kin); (b)DPM1 strain (MS + 0.25 NAA + 2 mg Kin); (c)control (B5 +0.5 2.4-D + 2 mg Kin); (d)control DP (LS + 0.5 2.4D + 2 mg Kin); (e)DPM1 strain (LS + 0.5 2.4D + 2 mg Kin); (f)DP(MS+0.5 mg/L 2-4D+2 mg/L Kin); (g)DPM1 strain (B5+0.5 mg/L 2-4D + 2 mg/L Kin); (h)DPM2 strain (B5+0.5 mg/L 2-4D+2 mg/L Kin).

(2 mg/L BAP + 0.5 mg/L NAA) resulted in the highest induction rate for somatic embryos (74%) in the MS medium, followed by the B5 medium with an induction rate of 73%; this was better than the rates for the other studied concentrations and media, with superiority found for the control and the DPM1 vegetative strain. The hormonal compatibility combination (1 mg/L BAP + 0.5 mg/L 2.4D) resulted in a 61% induction rate in the LS medium, making it superior to the other hormonal combinations in the same medium, with superiority found for the control, followed by the DPM1 vegetative strain. The interaction of auxins with cytokinins, in the 5C01 medium, enhanced the level of response to somatic embryo induction, but it was below the competitive level observed for the other studied media.

The results of this study agree with the results of Paul et al. (2012), showing the superiority of BAP supplemented with low concentrations of NAA in the MS medium, the results of Adsul et al. (2019) study on *Ceropegia mohanramii*, and the results of Verma et al. (2011) study on direct organ regeneration from explants of *Digitalis lamarckii Ivan*.

The superiority of NAA over both 2.4D and IAA when combined with BAP in the MS medium may be explained by the higher ability of the cells to absorb NAA. Moreover, the significant superiority of the nutrient media containing 2.4D compared with those with IAA was attributed to the side-chain effect for 2.4D; it was found that the nature and location of the substitution groups influence the activity of the compound, where the length of the side chain of the acetate group attached to the oxygen atom connected to the first carbon atom of the phenyl ring increased the activity of the auxin. The presence of two chlorine atoms attached to the second and fourth carbon atoms of the phenyl phenoxy acetic acid ring increased the activity and effectiveness of this auxin (Dayanandan, 1984).

The effect of the benzyl-adenine-ba-with-auxin interaction on the rate of direct-somatic embryogenesis induction

The results in Table 8 and Fig. 5 show that the hormonal compatibility combination (2 mg/L BA + 0.25 mg/L (NAA)) resulted in the highest induction rate of 81% in the MS medium, with superiority found for the control and

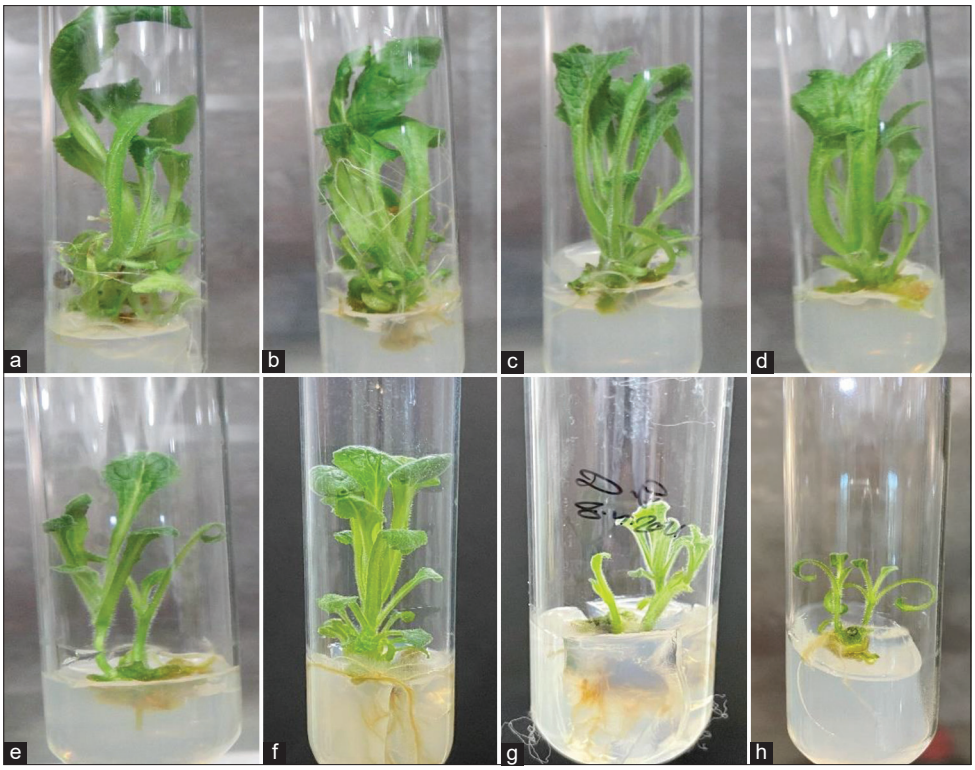

Fig 4. The effect of the BAP-with-auxin interaction on the rate of somatic embryo induction. (a)control (MS + 0.5 mg NAA + 2 mg BAP); (b)DPM1 strain (MS + 0.5 mg NAA + 2 mg BAP); (c)DPM1 strain (B5 + 0.5 mg NAA + 2 mg BAP); (d)DPM1 strain (LS + 0.5 mg 2.4D + 1 mg BAP); (e)DPM1 strain (LS + 0.5 mg 2.4D + 1 mg BAP); (f)DP (B5+0.5 mg/L NAA+ 2 mg/L BAP) ; (g)DPM2 strain (B5+ 0.5 mg/L NAA+ 2 mg/L BAP); (h)DP (5C01+ 0.5 mg/L 2-4D+ 1 mg/L BAP).

Table 8: The effect of the BA-with-auxin interaction on the induction rate for somatic embryos

		BA 1 (mg/L)						BA (2 mg/L)					
		2.4D		NAA		IAA		2.4D		NAA		IAA	
		0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5
MS	D.P	65	60	80	80	55	50	70	65	90	80	55	55
	DPM1	70	70	85	80	50	55	75	75	90	85	50	55
	DPM2	65	55	75	70	45	45	70	65	80	75	45	40
	DPM3	60	55	65	65	40	45	75	65	75	75	45	50
	DPM4	60	50	60	55	40	40	65	60	70	65	45	40
	Mean	64	58	73	70	46	47	71	66	81	76	48	48
B5	D.P	70	65	65	55	45	45	75	70	75	60	40	50
	DPM1	60	60	55	50	40	45	65	55	75	55	35	40
	DPM2	65	60	50	45	45	50	65	60	70	50	35	40
	DPM3	65	55	50	45	35	45	70	60	65	45	30	35
	DPM4	55	50	55	50	35	40	60	55	60	45	30	35
	Mean	63	58	55	49	40	45	67	60	69	51	34	40
LS	D.P	70	65	60	55	40	45	70	75	65	60	40	45
	DPM1	70	65	50	45	40	50	70	70	55	55	35	40
	DPM2	70	60	50	45	35	45	70	65	60	60	35	40
	DPM3	65	60	55	45	35	45	65	65	55	55	30	35
	DPM4	65	55	50	50	30	40	60	60	55	50	25	30
	Mean	68	61	53	48	36	45	67	67	58	56	33	38
5C01	D.P	45	45	45	40	30	35	40	45	35	40	30	35
	DPM1	40	30	35	30	20	25	30	35	35	30	20	25
	DPM2	35	25	30	25	20	25	30	35	30	25	15	20
	DPM3	25	20	20	20	25	25	20	25	30	25	15	20
	DPM4	25	20	20	15	15	20	20	25	25	20	10	15
	Mean	34	28	30	26	22	26	28	33	31	28	18	23

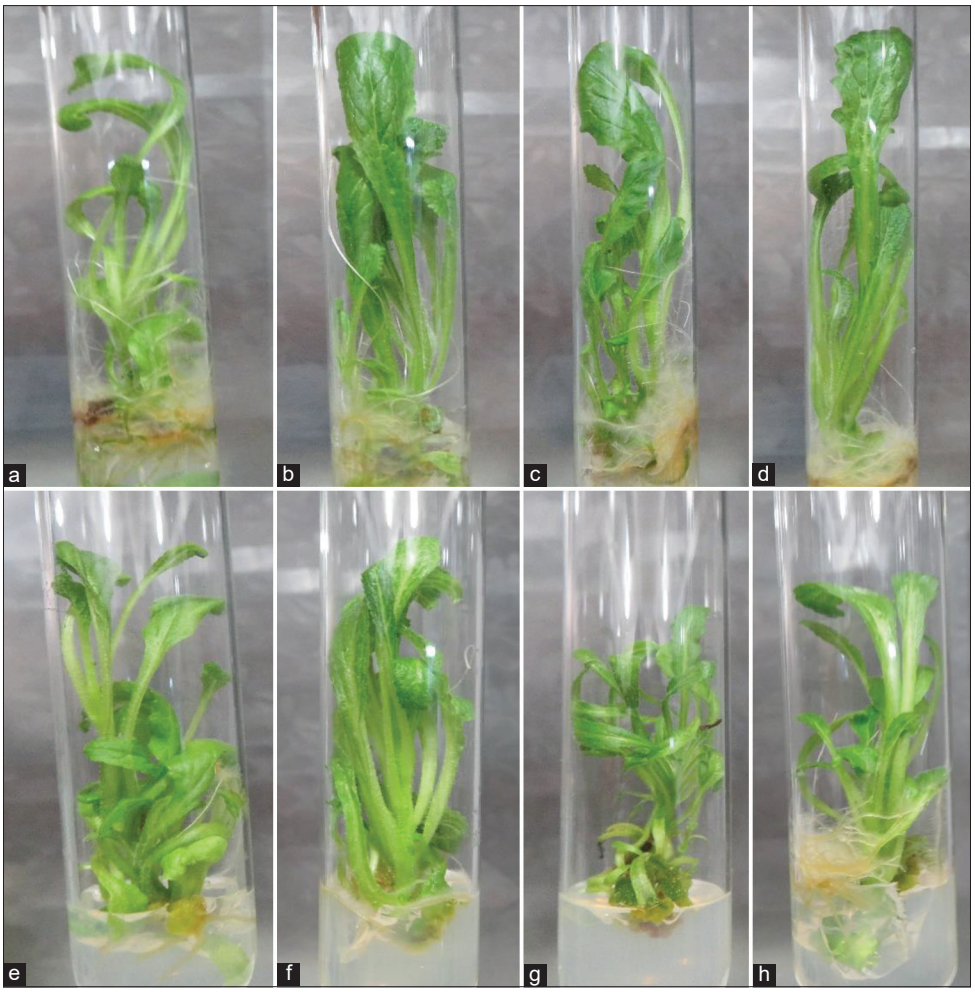

Fig. 5. The effect of the BA-with-auxin interaction on the rate of somatic embryo induction. (a)control DP (MS + 0.25 mg NAA + 2 mg BA); (b)DPM1 strain (MS + 0.25 mg NAA + 2 mg BA); (c)control DP (B5 + 0.25 NAA + 2 mg BA); (d)DPM1 strain (B5 + 0.25 NAA + 2 mg BA); (e)DPM1 strain (LS + 0.25 mg 2.4-D + 1 mg BA); (f)DPM3 strain (LS + 0.25 mg 2.4-D + 1 mg BA); (g)strain DPM4 (LS + 0.25 mg 2.4-D + 1 mg BA); (h)DPM2 strain (MS+0.25 mg/L NAA+ 2 mg/L BA).

the DPM1 vegetative strain, followed by the B5 medium, with an average induction rate of 69%; this was better than the rates for the other media and concentrations in the studied treatments.

Upon comparing the results in Tables 8 and 6, it was noted that the low concentration of NAA added to BA slightly enhanced the rate of induction compared to BA in single treatments, and this may be due to the state of synergistic induction that may be caused by the joint treatments in modifying or stimulating the levels of internal growth regulators.

This study agrees with the results of Youssef et al. (2019), showing the superiority of the interaction of NAA + BA in producing the largest number of plants when directly regenerating *Lilium orientalis c.v.*, and it agrees with the published findings by Krishnan et al. (2013) regarding the effect of BA supplemented with NAA in the MS medium in stimulating direct regeneration in rice strains *Oryza Sativa* L.; it also converges with the results of the same study for the B5 medium.

These findings are also similar to the published findings by Fatima et al. (2009) study on *Digitalis lanata Ehrh*, which confirmed the superiority of BA in direct somatic embryo induction compared with both Kin and BAP when interacting with auxins, and this may be because some cytokinins in the presence of auxins increased the response of the organism to certain growth regulators to a greater degree than others. However, the presence of both auxins and cytokinins was necessary for both to support their effects, as the activating concentration of one of them was ineffectual if it was alone. This can be explained by the different sites of action for auxins and cytokinins. The auxin works at the level of replication in DNA during the prophase, while the cytokinin works at a late stage in the cytoplasm division and during the telophase.

By comparing the results of Tables 6–8, it can be observed that our results agree with those of Hariprasath et al. (2015), which confirmed that low concentrations of auxins added to cytokinins were important for plant regeneration in the numbers of plant species, and the effects of the type and concentration of added plant-growth regulators on the growth of direct-somatic embryos varied according to the types, which was confirmed by Shen et al. (2018) on various types of orchids.

CONCLUSIONS

According to the findings of the current research work, it has been found that the induction phase, the direct induction rate, and the direct somatic embryo formation rate in the vegetative cultures of *D. purpuria* differed according to the culture medium type, the concentration and type of the plant-growth regulators, and the explant type, which differed in the potential-energy of their tissue-forming cells.

Also, it has been observed that using single cytokinins or very low concentrations of auxins in direct somatic embryogenesis induction is advantageous. Moreover, the preference of BA in direct somatic embryogenesis induction in the MS medium and BAP was superior in direct somatic embryogenesis induction in Ls and B5 media. Further, applying 5C01 medium indirect somatic embryogenesis induction was superfluous.

Author contributions

Mr. Mohammed Ahmed AL-Oqab has conceived and designed the experimental program, performed statistical analyses, interpreted results and wrote the original draft of manuscript. Dr Salim Zaid initiated this research and directed the entire experimental study. He also supervised the article redaction and interpretation of the results and edited the manuscript with the first author. Dr Youssef Al-Ammouri supervised the experimental data and revised the final paper. All the authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data presented in this study are available upon request from the corresponding author.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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