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EFFECT OF PHENYLALANINE, SORBITOL ON VEGETATIVE GROWTH AND PHENOLIC COMPOUNDS OF ORCHIDS IN VITRO.

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Abstract:

The study was conducted in the Plant Tissue Culture Laboratory of the Department of Horticulture and Landscape Engineering - College of Agricultural Engineering Sciences - University of Baghdad for the period from October 2021 to August 2022.with the aim of propagating the orchid plant *Phalaenopsis amabilis* (L.) tissue by determining the appropriate combination of growth regulators for all stages of propagation and studying the possibility of increasing the stimulation of some active compounds (phenolic compounds).By adding different concentrations of phenylalanine and sorbitol and comparing them with the same active compounds of the mother plant.As the single nodes were sterilized using commercial bleach (sodium hypochlorite) at a concentration of 15 ml for 15 minutes, then they were planted on MS germination medium supplemented with 60 ml L^{-1} coconut water, and then transferred on MS medium containing 2.5 mg L^{-1}BA and 1 mg L^{-1} NAA was added to it first with phenylalanine at concentrations of 0, 5, 10, 15 mg L^{-1}.Secondly, the addition of the sugar alcohol sorbitol at concentrations of 0,25,50,100 g.L^{-1} to increase growth and stimulate active compounds (phenolic compounds). As for phenylalanine acid, the results showed that phenylalanine had an effect on vegetative growth, where the concentration of 5 mg L^{-1} gave the highest rate of the number of shoots (4.5 shoots.explant^{-1}) and the number of leaves (5 leaves, explant^{-1}). As for the length of the shoot, the concentration was given as 10 mg L^{-1} A at a rate of (6.5 cm). As for its effect on the active compounds (phenols)The results showed that the addition of 10 mg L^{-1} of the acid gave the highest concentrations of compounds (Ellagic acid, Ferulic acid, Sinapicacid, p-Coumaric acid and Benzoic acid).As for the effect of adding sorbitol concentrations, significant effects were recorded on the number of shoot, as the comparison treatment and the concentration of 25 mg L^{-1} were significantly superior to the rest of the concentrations by giving them the highest rate of the number of shoot (4 shoots. explant $^{-1}$),As for the number of leaves, the comparison treatment gave the highest number of leaves, which reached 4 leaf .shoot-1, which did not differ significantly from the treatment 25 mg L^{-1} (3 leaf .shoot^{-1}), as for the effect of sorbitol concentrations on the active compounds.The results showed that the concentration of 100 mg L^{-1} was significantly higher by giving it the highest concentrations of all the studied phenolic compounds (Ellagic acid, Ferulic acid, Sinapic acid, p-Coumaric acid, and Benzoic acid).As for the comparison between the active compounds of field plants and the active compounds of tissue plants, the tissue plants were significantly superior to the compounds (Ellagic acid, Ferulic acid, Sinapic acid and p-Coumaric acid).

Keywords: phenylalanine, sorbitol, vegetative growth, orchids

Introduction:

The orchid plant belongs to the Orchidaceae family, which includes 736-889 genera, 27,800 species, and more than 100,000 hybrids worldwide (Cardoso et al. 2020) It includes monocotyledonous perennial herbs, the nature of its growth differs in different races. 85% of the species grow in areas close to the equator and between the Tropics of Capricorn and Cancer, and this means that they are spread in tropical and subtropical regions.Orchids grow in two forms, the first as epiphytic plants, where they grow on the shoot and stems of trees, and sometimes on rocks . As for the other picture, it represents terrestrial plants, and this group grows in the soil, where it obtains its food, like other plants, as in the genus Calanthe (Muradian, (1990).Orchids occupy a distinguished position in the trade of cut flowers in the world, and they are among the most beautiful and expensive cut flowers due to their longevity on the plant as well as their longevity in the vase, which reaches about a month (Amin et al., 2004).Many of them are also used as medicines because they contain phenolic compounds and antioxidants, where they play a role in preventing the formation of free radicals and relieving diseases such as cancer, autoimmune disorders, high blood pressure, atherosclerosis, and delaying aging (Wen et al., 2015) Orchids reproduce sexually by very small seeds, and one gram contains three million seeds. The germination of orchid seeds depends on the presence of certain fungi that live with seedlings in a symbiotic way. The mushroom threads grow between the growth environment and plant tissues to supply them with nutrients. This symbiotic life is necessary for orchids, especially in the early stages of growth.In order for the plants produced from the seeds to reach the size of the flowers, it takes several years, ranging between 5-7 years. It also reproduces by dividing or by successions for some species, or by non-true bulbs (false bulbs) (Mouradian, (1990) However, its cultivation in the field requires an area of land, service operations, and a relatively long time until the completion of plant maturity, in addition to the risks of field cultivation represented by climatic conditions and the unsecured environment, which negatively affects the growth and yield of these plants, and then the quantity and quality of active substances, and this prompted some researchers to use tissue culture technology in the propagation of orchids (Lal and Singh, 2021).Tissue culture technology has provided multiple opportunities for the production of secondary metabolite compounds continuously and without restriction to a specific season, and these compounds include phenols and the fact that the percentage of therapeutically active substances is low in most medicinal plants, which encouraged researchers to find ways to increase the active substances and encourage the plant to produce them by using some catalysts that contribute to their construction and the formation of primary or intermediate compounds that enter the structural pathways leading to the production of a specific compound (Karupusamy, 2009).Including the initiator Phenylalanine, which plays an essential role in the link between primary and secondary metabolism in plants, which is used as a protein building element and is an initiator of many plant compounds necessary for plant growth and development.

Ballica and Ryu (1993) showed that the content of alkaloids in cultures of D. stramonium callus increased by about five times more when the two amino acids Ornithine and Phenylalanine were added to the medium compared to the medium without them. Fett-Neto et al. (1995) reported that

the addition of phenylalanine to the culture medium of Taxus cupsidat stimulated the synthesis of anti-cancer compounds. Rosmarinic acid production can be stimulated from cell cultures of Salvia officinalis when 40 mg . $L^{-1}$is added of Phe to the medium compared to untreated medium (Karam et al., 2003)Where , Hamad (2011) indicated that the addition of amino acids to the food medium in vitro does not work as a precursor only, but as an elicitor as well. As the above researcher studied in his experiment on the plant Atropa belladonna, the effect of the amino acid Phenylalanine on the production of tropane alkaloids, The researcher summed up his results in using the amino acid Phenylalanine as a catalyst as follows, that the MS medium prepared with a concentration of 20 mg L^{-1} of the amino acid gave the highest rate of fresh and dry weight of callus, which reached 584.2 and 48.84 mg, respectively. As well as sorbitol (C6H14O6), which is one of the types of alcoholic sugars, as it acts as a source of sugars and can increase the osmotic pressure in the medium and thus lead to a decrease in water absorption by plant tissues.leading to a wide range of morphological and physiological changes in the tissues (Ramakrishna et al., 2011).Al-Marsoumi (2010) found that increasing the concentration of mannitol from 20 g L^{-1} to 40 and 60 g. L^{-1} led to an increase in the production of the active substance Thujone. It reached 161.9 and 157.2 micromoles gm-1 dry weight of callus, respectively, compared to the concentration of 20 gL^{-1} of mannitol which amounted to 125.4 micromoles g.L^{-1} in Saliva officinalis. Pa and Matthew, (2012) indicated that the addition of different concentrations of mannitol 0.80, 0.64, 0.32, 0.16 µmol L^{-1} to MS medium containing the suspension cells of Justicia adhateda plant gave the concentration 0.32 µmol L^{-1} the highest increase in the production of materials. The effective was 6.56 mg ml^{-1} compared to the neutral treatment. Mosleh and Abdul Rasool (2019) also found that the addition of the sugar alcohol mannitol at a concentration of 30 g. L^{-1} led to a significant increase in all studied traits in their study on the plant Capsicum annuum L.Based on the foregoing, the research aims to study the possibility of stimulating and increasing the production of active compounds (phenolic compounds) by adding different concentrations of phenylalanine acid and sorbitol and comparing them with the same active compounds of the mother plant.

MATERIALS AND METHODS

The study was conducted in the Central Laboratory for Plant Tissue Culture - College of Agricultural Engineering Sciences - University of Baghdad.

Multiplication stage

The single nodes were sterilized using commercial bleach at a concentration of 15 ml for 15 minutes (Al-Oubaidi and Jawad, 2021).Then, they were planted on MS growth medium containing 60 ml L-1 coconut water, and after 4 weeks, the vegetative growths resulting from the germination stage were transferred to MS medium equipped with a concentration of 2.5 mg L^{-1} BA by interfering with NAA at a concentration of 1 mg L^{-1} (Anuchai and Sasiangdee, 2020). The shoots abounded to be included in the two subsequent experiments.

The effect of phenylalanine on multiplying shoots and stimulating the active substance (phenolic compounds):

The resulting shoots were taken from the Multiplication stage and cultured on MS medium containing 2.5 mg L^{-1} BA and 1 mg L^{-1} NAA and supplemented with different concentrations of

Phenylalanine (0, 2.5, 5, 10) mg L^{-1} (Gawad & Ahmed, 2014).The results were taken after 6 weeks of cultivation, represented by the number of shoots, their lengths, the number of leaves, and the concentrations of the active substance.

The effect of different concentrations of sorbitol on multiplying shoots and stimulating the active substance (phenolic compounds):

The resulting shoots were taken from the multiplying stage and cultured on MS medium containing 2.5 mg L^{-1} BA and 1 mg L^{-1} NAA supplemented with different concentrations of sorbitol (0,25, 50, 100 (mg L^{-1}) (Açıkgöz, 2021). The results were taken after 6 weeks of cultivation, represented by the number of shoots, their length, the number of leaves, and the concentrations of the active substance.

Extraction of phenolic compounds:

Solid-liquid extraction is the most common procedure prior to the analysis of polyphenols and simple phenols in natural plants. They are still the most widely used technologies,This is mainly due to its ease of use, efficiency and wide application. Commonly used extraction solvents are alcohols (methanol, ethanol), acetone, ether,and ethyl acetate. The first steps of the preparation procedure is the grinding and homogenization process. Extraction is the main step to extract and isolate the phenolic substances present in the plants before the determination process.1.5 gm of the ground and homogeneous form is taken and 15 ml of chloroform and 10 ml of hexane are added to it in order to remove chlorophyll, terpenes and fats present in the plant and left for 10 hours with continuous stirring. Then the extract is placed in an ultrasonic cracker for 20 minutes at a temperature of 45 °C, after which 25 ml of methanol is added to it, then it is transferred to a separation funnel. Then the polar organic layer (methanol) is collected and transferred to the rotary evaporator to obtain a dry extract. The process was repeated 3 times to obtain a sufficient amount before analysis.

Analysis conditions:

The examination was conducted in the laboratories of the Ministry of Science and Technology - Department of Environment and Water, according to the method presented by (Minh et al. 2016) and (Abdul Husain and Jawad 2019) using a high-performance liquid chromatography (HPLC) model (Sykamn) of German origin.Where the carrier phase was used: methanol: distilled water: formic acid (70: 25: 5) and the separation column was: (C18 - ODS (25cm * 4.6 mm) to separate the phenols and an ultraviolet detector was used: UV - 280 nm, where the flow rate was The carrier phase is: 1.0 ml/min

Qualitative and quantitative determination of phenolic materials using the HPLC device:

Calculate the concentration of each sample using the following equation:

Unknown concentration (mg/g) = (Standard concentration x Sample area)/Standard area x (Number of dilutions/Sample weight)

The crops were incubated in the growth chamber under a light intensity of 1000 lux for 16 hours of light and 8 hours of darkness at a temperature of 25 ± 2 .

statistical analysis :

All experiments were analyzed using a completely randomized design CRD with one or two factors, 10 replicates for each treatment, and the results were analyzed using the Statistical Analysis System -SAS (2018) in data analysis to study the effect of different factors and their interactions on the studied traits.Significant differences between the means were compared with the Least Significant Difference-LSD test. In addition to the t-test at the 5% probability level (Al-Sahuki and Wahib, 1990).

Results and discussion:

Effect of phenylalanine acid on average number and length of shoot and number of leaves of orchids

The results in table (1) showed that the concentration of 5 mg L^{-1} was significantly excelled, giving it the highest average of the number of shoots, which amounted to 4.5 shoot, explant^{-1}, which did not differ significantly from the concentration of 2.5 mg L^{-1}.As it gave 4.2 shoot, explant-1while it differed significantly from the rest of the concentrations.As for the effect of phenylalanine acid on shoot length, the concentration of 10 mg L^{-1} was significantly excelled by giving it the highest average of shoot length of 6.5 cm compared to the comparison treatment that gave 1.5 cm, while it did not differ significantly from the rest of the concentrations.With regard to the number of leaves, the concentration of 5 mg L^{-1} was significantly excelled on the rest of the treatments by giving it the highest rate of 5 leaf.shoot^{-1} , which differed significantly from the treatment with a concentration of 10 mg L^{-1} that gave 2 leaf.shoot^{-1} leaves and did not differ from the rest of the treatments.

Table (1) Effect of the amino acid Phenylalanine on the average number of shoots (shoot.explant^{-1}) and the average length of shoots (cm) Average number of leaves (leaf.explant -1) of orchids grown on MS medium containing 2.5 mg L^{-1} BA and 1 mg L^{--1}NAA after 6 weeks of culture.

number of leaves (leaf.shoot^{-1})	shoot length(cm)	Number of shoots (shoot.explant^{-1})	Phenylalanine concentrations (mg L^{-1})
4	1.5	4	0
4	4.5	4.2	2.5
5	5	4.5	5
2	6.5	3	10
2.09 *	2.41 *	1.38 *	L.S.D
) *P≤0.05.(			

Effect of the amino acid Phenylalanine on the concentration of phenolic compounds of orchids:

Table (2) shows that the concentrations of the amino acid Phenylalanine had a significant effect on stimulating phenolic compounds in vegetative growths grown on MS medium supplemented with 2.5 mg L^{-1} of BA and 1 mg L^{-1} of NAA.The medium supplemented with 10 mg L- of the amino acid Phenylalanine gave the highest rate of weight than Ellagic acid, Ferulic acid, p-

Coumaric acid, Sinapic acid and Benzoic acid, which reached 284.7, 400.12, 90.56, 611.8, 23.6 µg.L^{-1}, respectively. Which was significantly excelled on control treatment, which gave an average of 232.14,325.65, 53.12, 12.55,512.25 µg g^{-1} - respectively. The results showed that phenylalanine has an effective role in improving vegetative traits in tissue culture, which can be explained by its effect on the biosynthesis of proteins.Amino acids are the building blocks for it, which regulate metabolism and transport and act as a storehouse of nitrogen.It also acts as a source of energy and biosynthesis of other organic compounds such as amines, purines, pyrimidines, vitamins, enzymes and terpenes, and is important for stimulating cell growth (Murphree, 2017).The reason for the increase in the quantitative estimation of phenolic compounds after the addition of the amino acid phenylalanine to the food medium may be due to its being the structural initiator of these compounds (Khanna et al., 2005), and it also enters through the shikmate biosynthetic pathway to manufacture various secondary compounds (Kliebenstein et al., 2001).This is consistent with what Al-Obaidi (2014) found, adding the amino acid phenylalanine at a concentration of 5 mg L^{-1} led to a significant increase in the concentration of rosmarinic acid compared to the control treatment and the field growth treatment.

Table (2) the effect of phenylalanine acid on the concentration of phenolic compounds µg g^{-1} in the shoots produced from the multiplication stage of orchids grown on MS medium containing 2.5 mg L^{-1} and 1 mg L-NAA after 6 weeks of culture.

Name	Control	2.5	5	10	$LSD_{0.05}$
Ellagic acid	53.12	62.58	70.11	90.56	**6.98 ***
Ferulic acid	325.65	348.99	366.98	400.12	**41.75 ***
Sinapic acid	512.25	536.9	574.8	611.8	**46.89 ***
p-Coumaric acid	232.14	250.6	266.9	284.7	**31.05 ***
Benzoic acid	12.55	15.9	18.9	23.6	**5.68 ***
*** (P≤0.05).**					

The effect of sorbitol on the average number of shoot, shoot length and number of leaves of orchids

Table (3) shows that sorbitol concentrations had a significant effect on the number of shoot, were the control treatment and concentration of 25 mg L^{-1} gave the highest average number of shoots, which reached 4 shoot.explant $^{-1}$.While the concentration of 100 mg L^{-1} gave the lowest average number of shoot, which reached 1 shoot.explant $^{-1}$, which did not differ significantly with the concentration of 50 mg L^{-1}.The same table showed the effect of adding sorbitol on shoot length as

the concentration of 50 mg L^{-1} gave the highest average shoot length of 3.5 cm, which did not differ significantly with the concentration treatment of 100 mg L^{-1}, while it differed significantly with the rest of the treatments.As for the average number of leaves, the comparison treatment was significantly excelled by giving it the highest rate of the number of leaves, which amounted to 4 leaves, $shoot^{-1}$, which did not differ from the concentration treatment of 25 mg L^{-1}, but differed significantly with the rest of the treatments.

Table (3) the effect of sorbitol on the average number of shoot (shoot.explant $^{-1}$), average length of shoot (cm), and average number of leaves (leaf.$shoot^{-1}$) of orchids grown on MS medium containing 2.5 mg L^{-1} BA and 1 mg L^{-1} NAA 6 weeks after culture.

number of leaves (leaf.shoot-1)	shoot length(cm)	Number of shoots (shoot.$explant^{-1}$)	**Sorbitol concentrations(mg L^{-1})**
4	1.5	4	**0**
3	1.3	4	**25**
2	3.5	2	**50**
1	2	1	**100**
1.77 *	1.86 *	1.72 *	**L.S.D**
) *P≤0.05.(			

Effect of sorbitol on the concentration of phenolic compounds of orchids

The results in table (4) show the effect of sorbitol concentrations on the phenolic compounds. The results showed that the concentration 100 mg L^{-1}, significantly excelled by giving it the highest concentrations of medicinal compounds Ellagic acid, Benzoic acid, Sinapic acid, Ferulic acid, and P-cumaric acid. It amounted to 66.59,348.59,540.25,255.98,15.15 µg $.g^{-1}$, compared with the control treatment that gave 53.12,325.65,512.25,232.14,12.55 µg $.g^{-1}$, respectively. Sorbitol reduces the osmotic pressure of the cell, which means a decrease in growth and limitation of cell size, as well as inhibition of cell division due to the difficulty in obtaining water and nutrients from the growth medium. This is reflected in the weakness of vegetative growth, represented by a decrease in the number of shoots and the number of leaves, and we do not miss mentioning the hormonal balance, which shifts toward the palm of growth inhibitors and reduces the effectiveness of growth stimuli in the presence of water stress due to sorbitol. These results are consistent with the findings of Al-Shammari (2018) when spraying with sugar alcohols (sorbitol and mannitol) on the plant Capsicum annuum L.The reason for the increase in the production of medicinal compounds when adding sorbitol may be due to the fact that alcoholic sugars are widely used in plant tissue culture for the purpose of increasing the osmotic potential.Which causes an increase in stress, leading to an increase in the formation of amino acid compounds, and thus enters through the biosynthetic pathways of Shikmate to manufacture various secondary compounds (Liu et al., Gautam, 2007, al-Khayri et al., 2011, and AL-Bahrany 2002).This is consistent with what was

found by Ibrahim and (Ameen 2017). It showed the possibility of increasing the amount of active compounds from the separated and cultivated explant in vitro when exposed to some stresses.As these active substances can be separated, purified and used in their pure form, as they are considered a natural source to be used in the preparation of the drug.

Table (4) The effect of sorbitol on the concentration of phenolic compounds in the shoots produced from the multiplication stage of orchids after 6 weeks of cultivation on MS medium.

Conc. / Name	0	25	50	100	$LSD_{0.05}$
Ellagic acid	53.12	48.59	60.15	66.59	7.24 *
Ferulic acid	325.65	300.15	328.97	348.59	31.67 *
Sinapic acid	512.25	495.79	511.58	540.25	35.93 *
P-cumaric acid	232.14	212.49	236.57	255.98	24.38 *
Benzoic acid	12.55	9.58	12.59	15.15	4.07 *
*** (P≤0.05).**					

Comparison between phenolic compounds of field plants and phenolic compounds of tissue plants

The results of Table (5) show the concentration of phenolic compounds in field plants compared with phenols in tissue plants.The results showed that the laboratory treatment was significantly superior to the field treatment for most compounds (Ellagic acid, Ferulic acid, Sinapic acid, and P-cumaric acid), while no significant differences were recorded for Benzoic acid.

Table (5) Comparison between phenolic compounds of field plants and phenolic compounds of tissue plants.

active substance	field plant	tissue plant	T-test
Ellagic acid	29.78	53.12	**6.02 ***
Ferulic acid	65.68	325.65	**37.58 ***
Sinapic acid	51.623	512.25	**41.66 ***
P-cumaric acid	28.53	232.14	**30.05 ***
Benzoic acid	31.49	12.55	**7.15 ***

* (P≤0.05)

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