The effect of lipo-polysaccharide isolated from *Sinorhizobium meliloti* bacteria on the tissue culture of *Pimpinella anisum* plants

[1]Najwa I.AL-Barhawi **[2]Raghad N.AL-Zaidy**
najwa_ibrahim@yahoo.com **raghadnawaf@yahoo.com**
Biology Deparetment , Education for Pure Science Colloge,Mosul University

ABSTRACT

Lipopolysaccharide was extracted from *Sihorhizobium meliloti* which is known for its symbiotic relation with legume plant *Medicago sativa*. This (LPS) was added to the following concentrations: (0.5, 1.0, 1.5 and 2.0)mg/l of solidified Murashige and Skoog (MS) medium, in addition to use other MS media containing mixtures of concentrations mentioned above. The (LPS) overlap with other concentrations of BA, and NAA were done to study its effect on callus initiation from roots, hypocotyls, and leaves of *M.sativa* and differentiation, in addition to examine its impact on weight soft for various explant callus and protein contents. It had been showed that the media (MS14 and MS15) were the best to stimulate callus formation from various explants in a rate of 100% with differentiation of the callus initiation to aerobic roots like structures, as shown with the combination of (MS15) a stimulation effect for (LPS) in callus differentiation of leaves and hypocotyls callus rates ranging (90-100)% with numbers ranging (25-30) branches within no more than (25) days and the combination of (MS13) was the best for stimulation of callus differentiation of root callus, which were (100)% with numbers (30) branches within 30 days. Results also had revealed an increase in the protein content of explants callus and regeneration in the media that supported by (LPS) with rates ranging (12-97, 74-120, 30-155)% and (43-77, 10-110, 25-118)% for callus of roots, hypocotyls, leaves and branches emerging respectively.

Keyword: *Pimpinella anisum*, *Sinorhizobium meliloti*, LPS, Tissue culture

INTRODUCTION

Anise plant (*Pimpinella anisum*) belongs to family *Umbelliferae*, and is considered one of the important medicinal plants as it contains volatile oils (1), phenolic ethers and flavonoids (2) and aniseeds compound known in its antifungal effect against fungus, bacteria and viruses (1).

Several studies had been conducted regarding the development and induction of callus from leaves and stems of anise plant, and the effect of adding different growth regulators in varying concentrations of 2.4-D and BA to the MS medium, which resulted high percentages of development rates (3,4).

On the other hand, the surface of the Rhisobium bacteria is characterized of its containment of different types of carbohydrate compounds of exo-polysaccharide (EPS), and many fatty sugars lipo-poly saccharides, polysaccharides, capsulas and β-1,2-glucan (5) that are known in their roles in the suppression of plant defense systems by protecting bacteria from total effect of plant toxins dispersed in the Rhizosphere area (6). LPS, which interferes the outer membrane structure of risobium bacteria is considered one of the specialized molecular markers and participates in all early and late stages of root nodes formation throughout the symbiotic relationship between these bacteria and leguminous plants (7) note that LPS compound found in *S.meliloti* bacteria is composed of four repeated units of glucose amino acid associated in β-1,4-linkage to form the N-acytyl-D-glucosamine chain (GLCNAC) and the sulfate group in the form of sulfated carbohydrates responsible for choosing its right legumes host (8).

A few studies have been conducted on the use of these carbohydrate compounds in field of tissue culture for some leguminous and non-leguminous plants, especially EPS compounds, which have been shown to have a clear catalytic effect in the development of callus from the parts of the *Helianthus annuus* L. flower plant, particularly when used in combination with BA in MS solid medium with a growth rate of 100% (9) , as well as the development of *Trigonella foenum graecum* in MS solid medium enriched in this compund with NAA and in development rates of 25, 50% with increased protein content (10). It also had an inductive effect in dividing seedling root cells of Fenugree plant not vaccinated with *S.meliloti* bacteria and forming its root nodules (11), as well as the use of polysaccharides LPS in the development of callus from the parts of the under-cotylednory stems and roots cuts of the clover Trifolum repens in MS solid medium at a concentration of 6 mg / L LPS isolated from bacteria Rhizobium *leguminosarium biovar phaseoli* (12).

Therefore, the present study was aimed to extract LPS from *S.meliloti* bacteria and study the possibility of its effect in stimulating the division of explant cells of Anise plant

Pimpinella anisum and the development and differentiation of callus interrelated with growth regulators commonly recognized for laboratory use in tissue culture of different plant parts.

MATERIALS AND METHODS

Isolation of *Sihorhizobium meliloti* bacteria and its growth conditions:

S.meliloti bacteria were isolated from the root nodeles formed on *Medicago sativa* planted at the wired house of the College of Education for Pure Sciences / Mosul University. The root nodeles with a part of root were cut and washed with running water several times to get rid of the lingering soil, then sterilized by immersing in ethyl alcohol (96%) for 1-2 minutes and washed with sterile distilled water (3-4 times) to remove alcohol residues. Then they were immersed in NaOCl (3%) solution and left in for 15 minutes and washed with sterile distilled water several consecutive times to get rid of the sterilizer effects and then transferred to sterile filter papers to remove excess of water. Then, it was distributed to nutrient agar surfaces and incubated for 24 hours at a temperature of 2 ± 30 ° C to ensure sterilization efficiency, and crushed with a little amount of saline solution. An amount of loop weight was transferred to surface of Yeast Extract Mannitol agar (YEM) to obtain isolated colonies after incubating for 24 hours at 2 °∓30 ° C in the growth incubator (13) , One colony was moved to the surface of the YEM medium in glass bottles and were incubated at the same conditions mentioned above for use in subsequent experiments and was replanted every (3-4) weeks to confirm their vitality before use.

Testing the susceptibility of *S.meliloti* isolated bacteria to the reformation of nodes on the roots of *Medicago sativa* plant experimentally:

The two-day seedling of *Medicago sativa* obtained from the Agricultural Research Centre of the Faculty of Agriculture were vaccinated by immersing its roots in 1 ml of a vaccine for previously isolated *S.meliloti* for 15 minutes. The seedlings were placed on the surface of the Nitrogen Free medium in the Petri dishes at a rate (5 seedling / dish) parallel to each other, and the dishes were wrapped with paraffin and kept vertically in the growth incubator (25∓2)C ° / 16 hours light / 8 hours darkness and intensity of lighting 3000 lux.

Extraction of (LPS) compound from *S.meliloti* bacteria:

The extraction procedure of LPS was according to (14) from the isolated *S. meliloti* bacteria, which was dried using the diluted pressure at the degree of extinction using the Lypholizer.

Viscosity measurement:

Aqueous solutions of lipo-polysaccharides isolated from *S.meliloti* bacteria were prepared at laboratory temperature (25-30 ° C) and measurement of viscosity was done by viscometer.

LPS colored chemical indicators:

A series of tests were conducted based on the method used by Robyt and White (15), hich included:

- Molish test.
- Acrolin test.
- Bial's test.
- Biuret test.

Determination of LPS components:

1- **Determination of glucose sugar in LPS:** The amount of glucose sugar in LPS was determined by following the method of (16).

2- **Determination of the chemical composition and structure of LPS:** a spectrophotometrically analysis was done to determine the active groups of LPS using the IR apparatus according to (12).

The development and differentiation of callus culture of different parts of anise plant *Pimpinella anisum* in MS medium supported by growth regulators and LPS:

Seedlings of *anisum pimpinella* were obtained with 30 days age after sterilization of seeds (obtained from the Agricultural Research Station of the Faculty of Agriculture and Forestry, University of Mosul) using the same sterilizers used for the root nodes mentioned before in MS solid medium (17).

Stems and roots were cut by 1 cm and 0.5 cm 2 leaves and then placed in glass vials on a 250 mL surface of the MS medium. The solid medium was supported with the following concentrations of growth regulators: SBA 1.5 mg / L and NAA 0.5 mg / L (leaves) and BA 1.0 mg / L and NAA 0.5 mg / L and NAA 0.5 mg / L (for stems and roots) (4).

Other containers of the same medium were supported by the following concentrations of LPS (0.5, 1.0, 1.5, 2.0) mg / L, as well as the use of other MS media containing a mixture of the above concentrations of growth regulators and LPS, and 4 pieces. All containers were incubated in the growth chamber under the lighting system, and the sequential darkness mentioned in the section on the germination of the germination

of the bacteria. After the development of the callus leaves, roots and stems of the Anise plantations, they were observed to examine its ability to differentiate and form the vegetative branches.

Sustaining the callus culture and estimate soft weight:

The cultivars were cultured every 4 weeks by removing the dead parts and dividing them into several pieces by weight (1 g / piece) and then placing them on the surface of the MS medium supported by the same concentrations of growth regulators and LPS. The weight was determined after 45 days of planting.

Determination of protein content:

A method of Lowry *et al*. (18) modified by (19) was used to extract and estimate the protein content of callus samples of different parts of Anise plant and its obtained branches. Bovin serum albomin was used as a standard solution in the standard curve drawing for those samples at a wavelength of 650 nanometer.

RESULTS

LPS compound that was isolated from *S.meliloti* bacteria had certain characteristics. Morphologically, it appeared white in color, fragile and it had the ability to dissolve in water forming a lipid solution with low viscosity (0.0019 sec / cm^2). Results obtained from chemical indicators' tests revealed its contents of carbohydrates and fats, but lack of nucleic acids and proteins. The results showed that it contained glucose content of 94.5%, fat by 1.7% and purity of 96.5%. From the other hand, the results obtained from spectrophotometrically analysis showed its absorption at wavelengths of (1700, 1800, 3500) cm -1, which indicated that this compound contains the carbonyl group, the ester group and the OH group (figures 1-a, and 1-b).

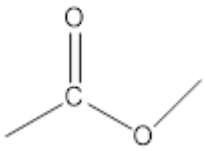

Figure (1-a): carbonyl group

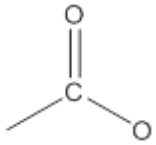

Figure (1-b): ester group

The MS medium used to develop the Anise plant *P.anisum* and supported with different concentrations (0.5-2.0) mg /L of LPS in the absence of sucrose, resulted in high seedling yield of this plant as well as the positive effect of increasing the total height rate of its vegetative content (table 1). The results showed the effect of this compound (LPS) on the development and differentiation of cultivars of different plant parts of *P.anisum* in association with growth regulators (NAA and BA). The MS15 and MS14 were distinguished by recording the highest percentage of 100% during close intervals (table 2), proven by division occurrence and development of callus completely after time periods ranged between (11-20) days. On the other hand, the LPS showed a clear effect on the formation of vegetative branches in different plant parts or callus in one step regeneration, especially with the MS4 medium supported with 0.2 mg / L of LPS, and its effect on the formation of 5 vegetative branches with percentage 50% during no more than 25 days, of callus leaves under study. However, the MS10, MS13 and MS15 cultivars encouraged the differentiation of developed leaves callus and formation of vegetative branches from 20-30 branches and 100% with a period of time not exceeding 26 days.

Table (1): Effect of LPS compound on the growth and development of anise plant *Pimipinella anisum* L seedlings

Medium No	LPS (mg/L)	Sugar (mg/L)	Growth %	Percentage of seeds forming the root mass	Percentage of seeds forming the vegetative mass	Period for initiation of growth
MSO	0.0	0.0	70	70	6.0	6
MS	0.0	30	80	80	80	6
MS1	0.5	0.0	100	100	100	4
MS2	1.0	0.0	85	85	85	6
MS3	1.5	0.0	100	100	100	5
MS4	2.0	0.0	90	90	90	4

MSO = MS solid sugar – free medium (negative control sample).
MS1 = MS sugar-fortified solid medium (positive control sample).
MS2 = MS sugar-free solid, 0.5 mg / LPS fortified medium.
MS3 = MS sugar-free solid, 1.5 mg / LPS fortified medium.
MS4 = MS sugar-free solid, 2.0 mg / LPS fortified medium

Table (2): Development and differentiation of callus of leaves for anise plants *Pimipinella anisum* L. in MS solid medium with different concentrations of NAA, BA and LPS

Medium No.	Media contents			Callus development		Callus differentiation		
	LPS	NAA	BA	Day	%	No vegetative branches	Branching formation %	Period (day)
Co-	0.0	0.0	0.0	-	-	4	25	8
Co+	0.0	0.5	1.5	20	65	5	20	24
MS1	0.5	0.0	0.0	-	-	-	-	-
MS2	1.0	0.0	0.0	-	-	-	-	-
MS3	1.5	0.0	0.0	-	-	-	-	-
MS4	2.0	0.0	0.0	-	-	15	50	25
MS5	0.5	0.5	0.0	40	100	-	-	-
MS6	1.0	0.5	0.0	20	40	-	-	-
MS7	1.5	0.5	0.0	-	-	-	-	-
MS8	2.0	0.5	0.0	-	-	-	-	-
MS9	0.5	0.0	1.5	-	-	-	-	-
MS10	1.0	0.0	1.5	20	75	20	100	22
MS11	1.5	0.0	1.5	-	-	-	-	-
MS12	2.0	0.0	1.5	-	-	-	-	-
MS13	0.5	0.5	1.5	22	60	30	100	18
MS14	1.0	0.5	1.5	-	-	10	40	26
MS15	1.5	0.5	1.5	20	100	30	100	25
MS16	2.0	0.5	1.5	41	100	20	50	50

The results in table (3) showed that the interference from MS9 and MS14 was greater than 100% in the development of callus from the stems over a period of 15-20 days followed by MS5 by 90%, as well as its effect on the differentiation and composition of the vegetative content by 90-100% in both medium MS15 and MS16 respectively, as recorded above Callus formation of vegetation on its surfaces evolved after 27-35 days of agriculture to branches of vegetables and one stage on solid MS-supported (0.5 NAA, 1.0 and 1.5 LPS) mg / L with observed formation of aerobic-like structures, MS (0.5 mg / LA and 1.0 mg / L BA) mixed with 0.5, 1 and 1.5 mg / L LPS compound, after different periods of cultivation of these pieces on the mentioned media (figure 2).

Table (3) Effect of LPS in the development and differentiation of stems callus of anisum plants *Pimipinella anisum* L. in MS solid medium

Medium No.	Nutrient Media contents			Callus development		Callus differentiation		
	LPS	NAA	BA	Day	%	No vegetative branches	Branching formation %	Period (day)
Co-	0.0	0.0	0.0	-	-	3	10	33
Co+	0.0	0.5	1.5	33	70	23	85	56
MS1	0.5	0.0	0.0	-	-	-	-	-
MS2	1.0	0.0	0.0	35	60	10	70	40
MS3	1.5	0.0	0.0	-	-	25	80	37
MS4	2.0	0.0	0.0	-	-	15	80	41
MS5	0.5	0.5	0.0	37	90	2	50	14
MS6	1.0	0.5	0.0	20	25	5	20	20
MS7	1.5	0.5	0.0	-	-	-	-	-
MS8	2.0	0.5	0.0	22	25	-	-	-
MS9	0.5	0.0	1.0	20	100	10	80	8
MS10	1.0	0.0	1.0	-	-	-	-	-
MS11	1.5	0.0	1.0	-	-	-	-	-
MS12	2.0	0.0	1.0	-	-	-	-	-

Medium No.	Nutrient Media contents			Callus development		Callus differentiation		
	LPS	NAA	BA	Day	%	No vegetative branches	Branching formation %	Period (day)
MS13	0.5	0.5	1.0	20	100	30	80	20
MS14	1.0	0.5	1.0	8	100	6	100	8
MS15	1.5	0.5	1.0	15	100	25	90	17
MS16	2.0	0.5	1.0	17	40	-	-	-

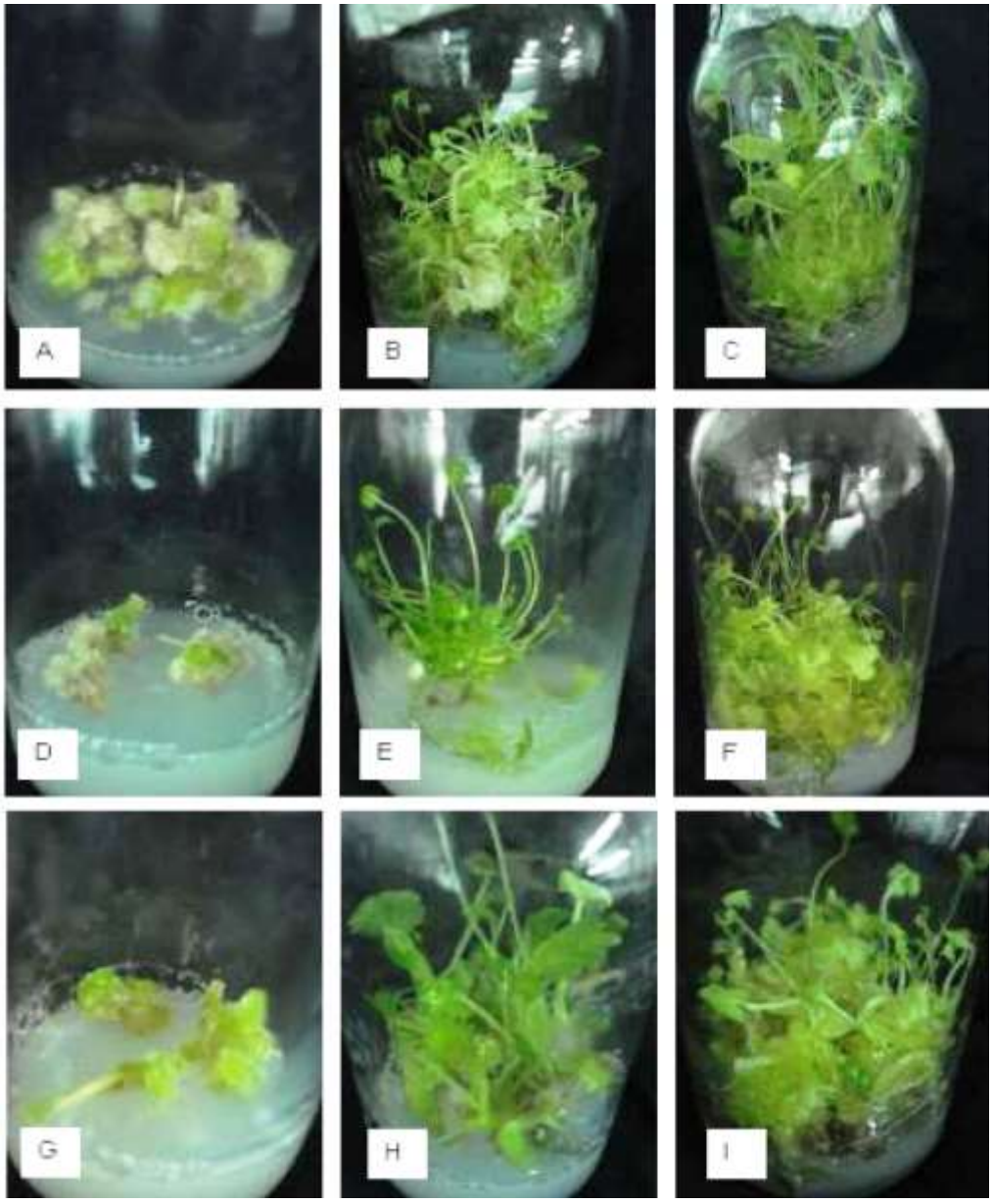

Figure (2): The development and differentiation of the callus of different parts of *Pimpinella anisum* L in MS solid medium supported by the LPS: A: The development of callus leaves in MS15, B: Differentiation of callus leaves in MS15, C: Differentiation of callus leaves in MS13, D: Introducing callus stems in MS14, E: Differentiation of callus in MS14, F: Differentiation of callus stems in MS14, G: initiation of root callus in MS15, H: Differentiation of the root callus in MSl3, I: Differentiation of root callus in MSl4

On the other hand, it was observed that the best cultivars for developed callus of different parts of this plant were in MS with the following concentrations (1.5 LPS, 0.5 NAA, 1.5 mg BA) mg / L, 1.5 LPS, 0.5 NAA and 1.0 BA (0.5 LPS, 0.5 mg NAA and 1.0 mg BA) / L (table 4) for each of the leaves, stems and roots respectively, due to callus ability to sav its vitality as it continues to grow each month when the start of appearance of brown parts and the dryness and cracking of the medium (figure 3).

Table (4) Effect of LPS in the development and differentiation of root callus of anisum plants *Pimipinella anisum* L. in MS solid medium

Medium No.	Nutrient Media contents			Callus development		Callus differentiation		
	LPS	NAA	BA	Day	%	No vegetative branches	Branching formation %	Period (day)
Co-	0.0	0.0	0.0	56	100	8	40	80
Co+	0.0	0.5	1.5	8	100	18	70	30
MS1	0.5	0.0	0.0	-	-	-	-	-
MS2	1.0	0.0	0.0	-	-	1	20	42
MS3	1.5	0.0	0.0	-	-	-	-	-
MS4	2.0	0.0	0.0	-	-	-	-	-
MS5	0.5	0.5	0.0	14	100	1	100	27
MS6	1.0	0.5	0.0	30	10	-	-	-
MS7	1.5	0.5	0.0	20	100	-	-	-
MS8	2.0	0.5	0.0	-	-	-	-	-
MS9	0.5	0.0	1.0	-	-	-	-	-
MS10	1.0	0.0	1.0	-	-	-	-	-
MS11	1.5	0.0	1.0	-	-	-	-	-
MS12	2.0	0.0	1.0	-	-	-	-	-
MS13	0.5	0.5	1.0	8	100	30	100	30
MS14	1.0	0.5	1.0	8	100	12	90	30
MS15	1.5	0.5	1.0	10	100	31	100	35
MS16	2.0	0.5	1.0	32	50	-	-	-

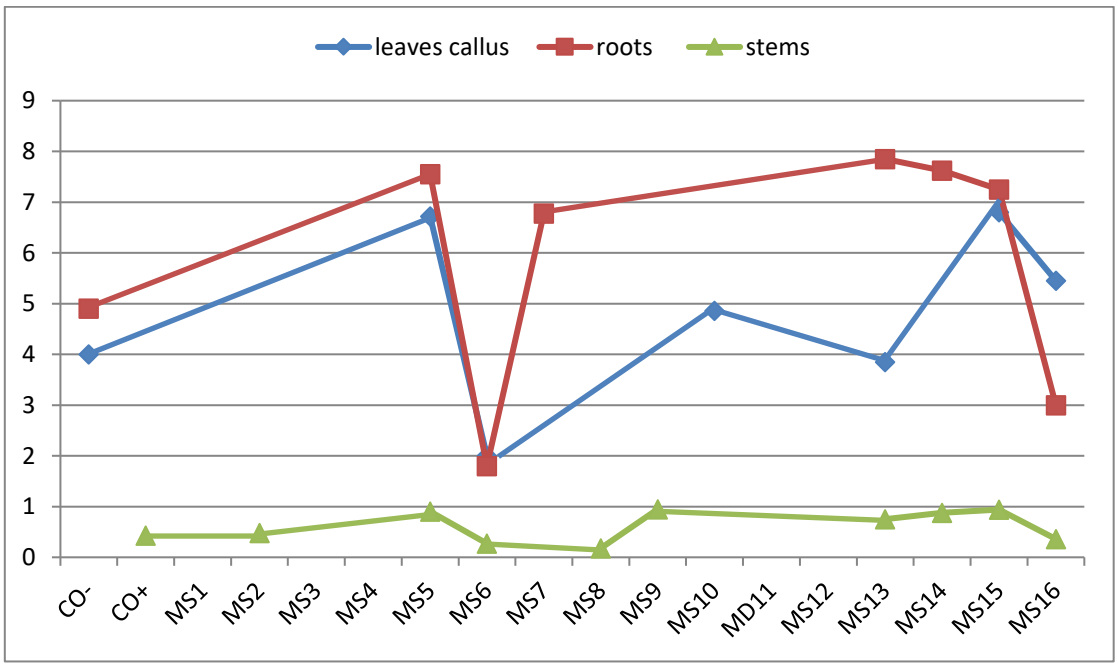

Figure (3): Soft weight ratios for callus in plant parts (gm) in Ms media supported by different concentrations of growth regulators and LPS

The protein content of the plant parts culture and the resulting vegetation was increased in the LPS-supported nutrition media compared to non supported medium, with an increase of 12-97, 74-120, 30-155%, and 43-77, 10-110, 25-118%) in each of the callus of root, subapical stems, leaves and their resulting branches (figures 4,5,6 respectively).

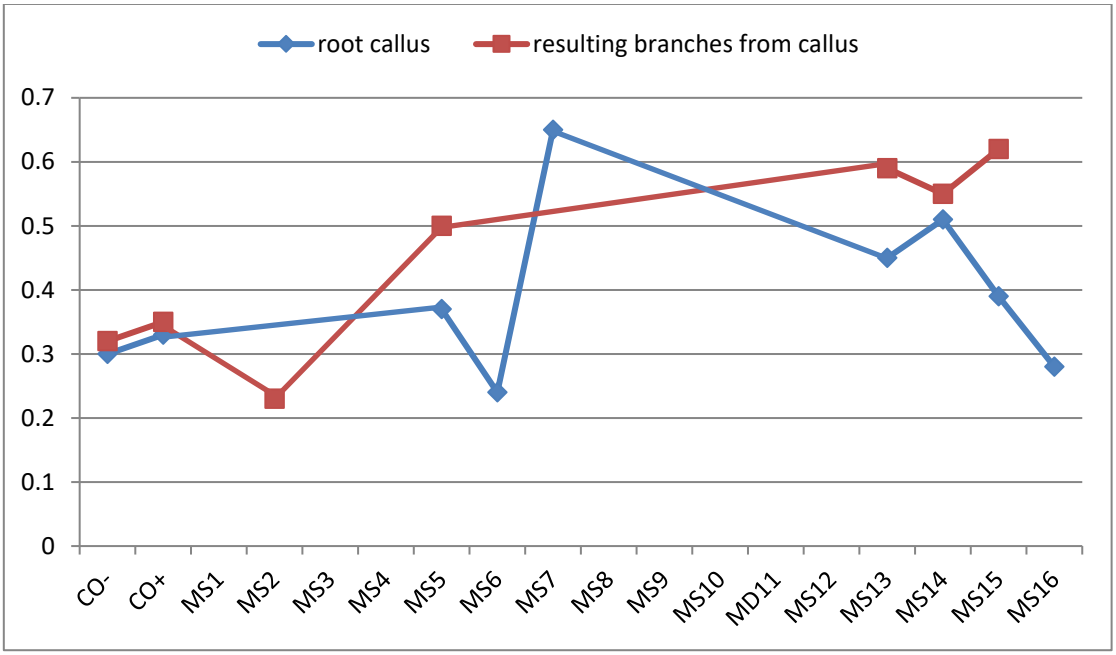

Figure (4): The rates of protein in the root callus and the branches produced from callus in Ms media supported by different concentrations of growth regulators and LPS

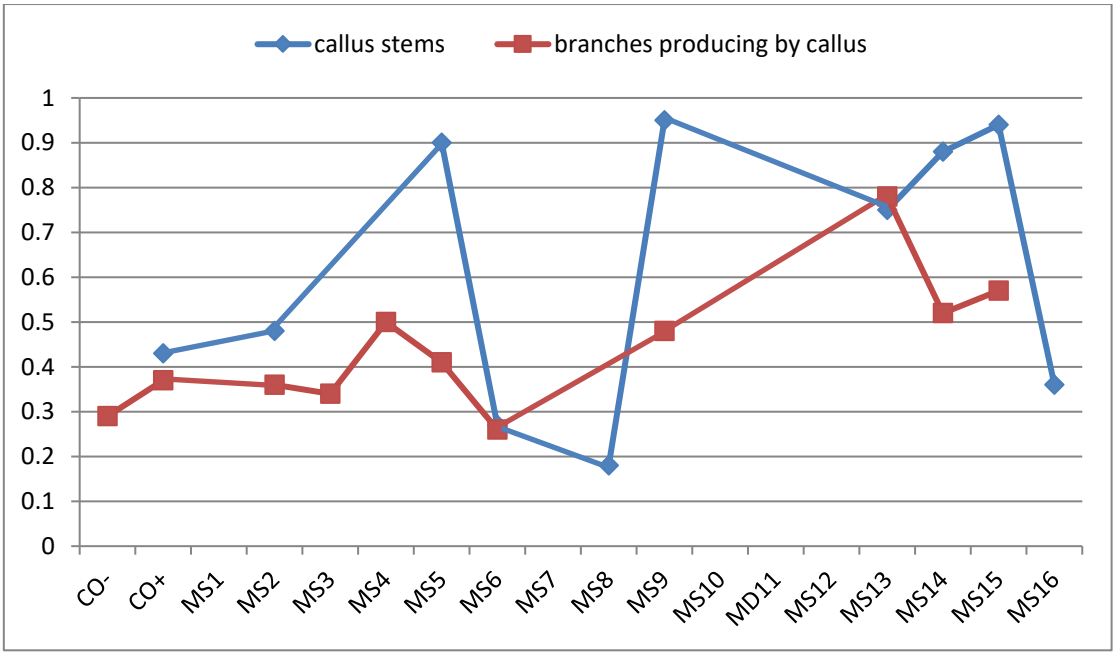

Figure (5): rates of protein contents in Stems and its branches in Ms Supported by Different Concentrations of Growth Regulators and LPS

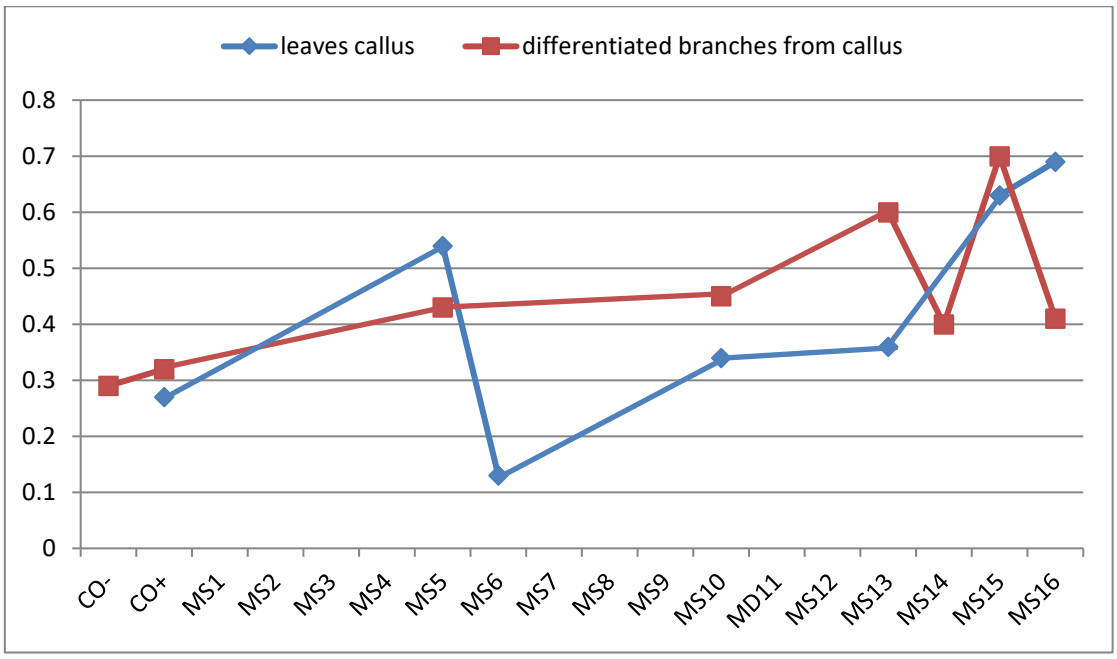

Figure (6): Rates of protein l in leaves callus and differentiated plants from callus in MS media supported by different concentrations of growth regulators and LPS

DISCUSSION

The study of microbiology and its ability to produce various compounds was the focus of many scientific researches, as well as the study of its importance to its producing organism from fungus, algae or bacteria (20).

In the present study, LPS production from *S.meliloti* isolated from the root nodeles of Alfalfa plant was studied, and detected its ability to produce this compound throughout the results obtained from using chemical indicators and tests, which gave positive detection in containing carbohydrates and fats and free of nucleic acids and proteins, in addition to the results of spectrophotometrically analysis by IR, which proved that the substance derived from the bacteria *S.meliloti* is LPS, in terms of containing groups of: Carboxyl, Ester and hydroxyl (21).

The contents of carbon and nitrogen sources of the LPS compound isolated from *S.meliloti* bacteria represented in repeated units of glucose Amin (22), when added to MS carbon-free solid media had increased the growth rate of the anise plants, as well as the increase in their vegetative growth rates.

On the other hand, the results showed the effective role of this compound (LPS) when used separately or in combination with different growth regulators in the field of tissue culturing of different parts of anise plant *P.anisum* under study, especially when compared with the development in MS solid medium supported by (0.5 mg / NA) and NA (0.5) mg / l, as the best combinations used for the development of callus for roots, stems and leaves respectively. This may often be due to the occurrence of hormonal imbalance between inside and outside the cell (23).

On the other hand, a distinct success of the LPS compound was observed when interfering with NAA in the development of callus and increasing its protein content in different parts of the plant, while failed when adding BA. This is explained by assuming the behavior of this compound such as cytokinein in the presence of sufficient amount of oxygen, encourage the mitotic division of plant cells (24) and provide hormonal balance requirements both inside and outside cells (25).

It also led to the doubling of DNA with the abundance of RNA needed for development (26) because the pathway of genetic information begins with DNA transucrption and mRNA and its translation into protein (27), as well as the presence of pyruvate, sucinate and acetylate organic acids in LPS (28), which inevitably interfere with the groups of essential amino acids to form and increase protein compounds within plant cells (29). In addition, LPS has a similar effect of vitamin C in the clearance of cells from many of the inhibitory compounds to maintain the vitality of callus and continued divisions (30). However, LPS failure to develop callus of roots and leaves when added alone despite its behavior as a growth regulator (31) may be due to the insufficient concentrations used or to the self-containment of these plant parts on sufficient cytokines and the low content of oxygen, which explains the incomplete requirements of the plant cell cycle that usually is affected by the presence of these two regulators (24), noting the high rates of development of

callus in this study when compared with the development ratios of these parts with the LPS compound, different NAA were mixed with different combinations of growth regulators (4).

The differentiation of some plants or their callus into vegetative branches in different quantities may be due to the important role of the balance between the addition of LPS with the growth regulators, the genetic composition of the cultivated plant parts or the physiological conditions of the plant from which the plant part is taken (32) ,or the type and origin of plant parts and environmental conditions during agriculture (33).

Another evidence of the effectiveness of this compound in the field of tissue culture when added to MS solid media supported by the growth regulator NAA, and the development of cells Callus to structures or clusters of green cells on the surfces and then to the vegetative branches within a month of cultivating plant parts, and leaves at a single stage, while MS-supported growth regulators, such as oxygen and cytokines, were introduced in the development of callus and their differentiation into vegetative branches during two different stages of time and their content of quality and concentration of growth regulators (34).

In the future, several studies can be carried out on the use of LPS in the tissue culture of plants belonging to other families and its use in association with other growth regulators to obtain its effects and determine its biological behavior in various tissue culturing fields.

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