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Induced callus from seedlings of *Peganum harmala* L. and studying harmine compound concentration *in vitro* and *in vivo* by GC analysis.

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Abstract

Plant tissue culture considers a benefit biotechnological technique for scientific research especially the production of undifferentiation callus cells and regeneration through suspension or static media. The seedlings of *Peganum harmala* was used as a source to produce callus mass *in vitro* in static media through different tissue culture media supplemented by varying combinations of plant growth regulators (PGR). The result illustrates that 2 mg/l of Kinitine with 0.5 mg/l of 2, 4-D was efficient to stimulate callus induction with percent 100% in stem and root of *P. harmala* and this combination gave a high fresh weight, 1954 mg in root and 1170 mg in stem and high dry weight in root and stem was 74.60, 60.30 respectively. In a comparative analysis through gas chromatography (GC) the stem and root in field recorded harmine concentration 56.13 and 40.95 µg respectively, which was higher than the *in vitro* callus induction from stem and root, which may be due to the fact that field plants have not been exposed to plant hormones with concentrations higher than the normal level, which reduced the stimulation of cells producing active compounds.

Keywords: *Peganum harmala*, plant growth regulators, callus, Kinitine, 2, 4-D, harmine, *in vitro*, GC.

استحثاث الكالس من بادرات نبات الحرمل و دراسة تركيز مركب الحارمين بواسطة كروماتوكرافيا الغاز داخل و خارج الجسم الحي

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الخلاصة

تعتبر تقنية زراعة الانسجة النباتية من التقنيات المفيدة والهامة في مجال البحوث العلمية خاصة في انتاج خلايا الكالس غير المتمايز والإخلاف من خلال المعلقات الخلوية او الأوساط الزرعية الصلبة. تم إستخدام بادرات نبات الحرمل كأجزاء نباتية لإستحثاث الكالس خارج الحسم الحي على أوساط زرعية صلبة و بتوليفات مختلفة من هرمونات النمو النباتية. وقد اوضحت النتائج بأن الكاينتين بتركيز 2 ملغم/لتر مع 2,4-D بتركيز 0.5 ملغم/لتر كانت فعالة في استحثاث الكالس و بنسبة 100% لجذر وساق نبات الحرمل. كذلك هذه التوليفة من الهرمونات اعطت أعلى وزن طري بلغ 1954 ملغم في الجذر و 1170 ملغم في الساق بالإضافة الى تسجيله أعلى وزنا جافا في الجذر والساق بلغ 74.60 و 60.30 ملغم على التوالي. وبيّن التحليل المقارن لتقنية كروماتوكرافيا الغاز بين ساق وجذر النبات ان هنالك إختلافات في تركيز مركب الحارمين خارج وداخل الجسم الحي فقد بلغ أعلى تركيز للحارمين على التوالي 56.13 و 40.95 مايكروغرام عند الساق والجذر المزروع حقلياً وكان أعلى من الكالس المستحث خارج الجسم الحي والذي قد يكون بسبب ان النباتات

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الحقلية لم تتعرض الى الهرمونات النباتية بتراكيزاعلى من المعدل الطبيعي ادت الى تقليل التحفيز لدى الخلايا المنتجة للمركبات الفعالة.

Introduction

Peganum harmala is a medicinal plant belonging to Nitrariaceae family, distributed in Iraq in middle and southern areas in dry soil and deserts [1], a wild perennial plant blossoms between June and August in the Northern Hemisphere [2].The phytochemical screening and qualitative analysis of *P. harmala* led to the isolation of different types of chemical ingredients such as tannins, steroids, sterols, saponins, flavonoids, anthraquinones, amino acids, polysaccharides and alkaloids mostly β-carbolines, such as harmine, harmaline, harmalol, and harmol from its seeds, leaves, flowers, stems and roots [3, 4]. The β-carbolines alkaloids the main substances responsible for the antimicrobial activities, antidepressant, antinociceptive, analgesic, antioxidant, antitumor and vasorelaxant activities of *P. harmala* [4, 5], also have effects on both central and peripheral nervous system and to treat Parkinson's disease [6], cardiovascular effects, antimicrobial agent and anti-leishmanial remedies [7] [8] or probably treatment of genital herpes HSV-2 as antivirus [9] and have antioxidant activity [4]. Plant callus is a growing mass of undifferentiated parenchyma cells formed according to outer tissue injures in living plants. In biotechnology research callus initiation *in vitro* according to planting intact and sterilizing plant tissue onto induction medium supplement with regulatory phytohormones such as auxins, cytokinins, and gibberellins [10]. Plant tissue culture consider a benefit technique for production of secondary metabolites from callus in this study would focus on *P. harmala* seedling using as material for callus induction. In this study Gas chromatography techniques (GC) was used to determine the quantity and quality of harmine alkaloid between stem and root in *P. harmala* plants *in vivo* and *in vitro*.

Materials and methods:

Preamble *In vivo* planting in field:

The Seeds of *P. harmala* supplied from local market in Baghdad were divided into two parts, one part for *in vivo* planting (field), the other for *in vitro* culturing. The field culturing by preparation of pots 30 cm in diameter filled with ordinary soil with manure in equal amount 1:1 ratio. The pots were kept in house garden until maturity after 50-55 days. The field plantlet yield for comparison with laboratory sample

The preparation and sterilization of seeds culture medium:

Half strength Murashige & skoog (1962) MS medium ready-made from Himedia Producer Company. The PH was adjusted to 5.8 using 0.1 NaOH or 0.1 HCl, then 7 g/l agar was added to the medium. The volume of medium was completed to 1 liter. The medium was dissolved by heating on a hot plate magnetic stirrer till boiling. The medium was dispensed equally into cultural tubes (50 ml/tube). They autoclaved at 121 C˚ under pressure of 1.04 kg/cm² for 15 minutes and then allowed to cool at room temperature.

The seeds surface sterilization and seeds viability *In vitro* test:

The seeds put under running tap water for 60 minutes, then soaked in 70% (v/v) ethanol for 30 seconds with stirring, ethanol alcohol consider efficient and powerful sterilizing agent due to its higher polarity than absolute ethanol which penetrates the cell membrane of the microorganisms [11]. The seeds then washed by distilled water three times for 5 minutes to remove the alcohol remnant. The completed sterilization operation by socked the seeds with different concentrations of sodium hypochlorite (1.5, 3, 4.5, 6) % for 5, 10, or 15 minutes, then all seeds washed with sterile distilled water three times for 5 minutes each. Sterilized seeds were cultured under aseptic conditions in laminar air flow hood on MS medium without growth regulators in plastic bottles. Ten seeds were cultured in each bottle with 3 replicates for each treatment. All the cultures were placed in lighten growth room at 25±20 C° with 16/day photoperiod with light intensity 1000 lux provided by cool white fluorescence lamps [12]. After 19 days of seeds induction the germination percent (%) was calculated using the formula [13]:

$$Germination\ percentge = \frac{\text{numer of } germination\ seeds}{\text{number of total cultured seeds}} \times 100$$

The preparation of callus induction and maintenance medium:

Ten types of MS media supplemented with different combination of plant growth regulators were used for callus induction as lighten in Table-1. Seedlings grown *In vitro* were used as explants source.

Roots, stems, cotyledons, and true leaves (Figure-1) were chosen for culturing on basal MS medium containing different combinations of phytohormones [14]. The four explants were cultured in 10 replicates by putting in universal tubes and incubated in the growth room at 25±20 C° with 16/day photoperiod with light intensity 1000 lux. The weekly examination after 4 weeks, the callus began to be observed and the callus induction percentage (Cip) was calculated by following formula [15]. The chosen of maintenance media according to the highest callus induction percentage.

$$Cip = \frac{\text{number of explants forming callus}}{\text{total number of cultured explants}} \times 100$$

Table 1- The composition of callus induction media used for *P. harmala*

Media codes	Modified MS-media composition
MS_0 (control)	MS salts (4.9 g/l) + Sucrose (30 g/l) + Agar (7 g/l) free hormones
MS 1	MS_0 + BA (0.5 mg/l) + NAA (0.5 mg/l)
MS 2	MS_0 + BA (2 mg/l) + NAA (0.5 mg/l)
MS 3	MS_0 + Kin (0.5 mg/l) + NAA (0.5 mg/l)
MS 4	MS_0 + Kin (2 mg/l) + NAA (0.5 mg/l)
MS 5	MS_0 + BA (0.5 mg/l) + 2,4-D (0.5 mg/l)
MS 6	MS_0 + BA (2 mg/l) + 2,4-D (0.5 mg/l)
MS 7	MS_0 +Kin (0.5 mg/l) + 2,4-D (0.5 mg/l)
MS 8	MS_0 +Kin (2 mg/l) + 2,4-D (0.5 mg/l)
MS 9	MS_0 + NAA (0.5 mg/l)
MS 10	MS_0 + 2,4-D (0.5 mg/l)

The measurement of callus fresh & dry weight

The callus fresh weight was measured after four weeks of explants culturing onto induction medium by cleaning the callus before sub-culturing from the medium and putting it on a filter paper then measuring it by sensitive electronic balance. The fresh callus was dried in the oven at 60 C° for 48 hrs. The callus dry weight was taken after the steady of fresh weight

The qualitative and quantitative analysis for harmine alkaloids by GC-analysis

The chemical compound harmine alkaloid was diagnosed by used Gas chromatography (GC) model SHIMADZU 2010. The separation column type DB5 with 30 mm × 0.25 mm × 0.25 mm dimensions. The temperature degree began in 90c reached to 220 C° gradually increasing by 10 C°/min. while the temperature of injection area and detector area are 280 and 340 C° respectively. In the detector area the indicator type wasthe flammable ionized detector for active compound indication, the nitrogen N was used as transporter gas. The concentration account was depending on retention time and sample area according to formula [16].

$$[sample] = \frac{[\text{St.}] \times \text{A. sam.}}{A.St.} \times \frac{\text{DF}}{\text{W. Sam.}}$$

[Sample] = sample concentration, [St.] = standard concentration, A. Sam. =Area of sample, A.St. = Area of standard, DF = dilution factor, W. Sam. = weight of sample.

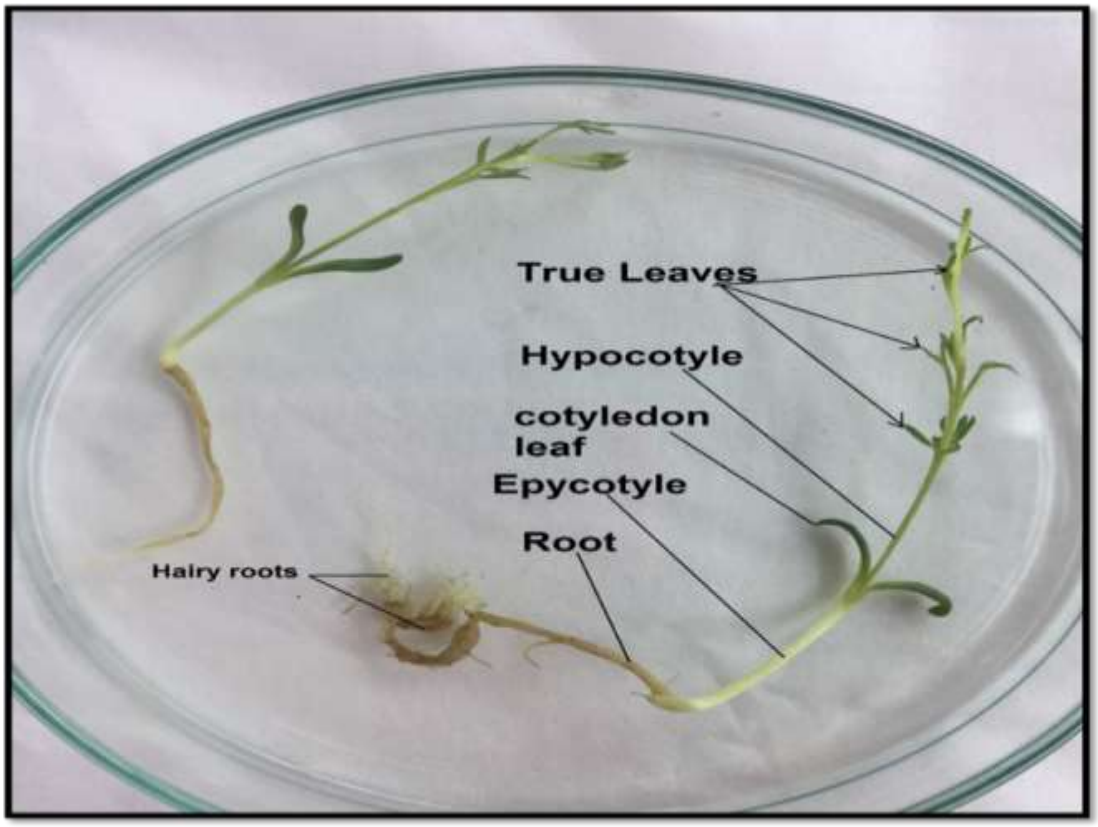

Figure 1-*P. harmala* seedling at laboratory culture after 19 days

Statistical analysis:

The experiments were implemented by augmented factorial in completely randomized design (CRD). The data were analyzed by GenStat software program V. 12.1, and the means were compared by the least significant difference (LSD) at (0.05) [17].

Results and discussion

The surface sterilization of the seeds is a normal manner in plant tissue culture to get rid of as possible all contamination factors that disturb the culture process.

Table-2 recordeda high percent 72.2% of seeds germination appear in 6% concentration of sodium hypochlorite which was not significantly different with 1.5% that gave 71.1% of vivid seeds emergence to seedlings. The treatment 6% and 1.5% significantly difference with 3% and 4% treatments. On the duration factor, the 5 and 10 minutes show no significant differences and gave an acceptable germination percent 71.7% and 68.3% respectively.

The interaction analysis between the percentages of sodium hypochlorite and sterilization time in Table-2 showed that the lowest percentage of vivid seed germination was 50% in 5 minutes. exposure to sterilizing solution in treatment 3%, and a high percent was 90% in 6% treatment for 5 minutes duration. The increasing of sterilizing duration in 6% treatment may decrease the germination of seeds due to its toxicity [18].

Bleach solution contains 6% sodium hypochlorite consider active disinfecting agent and widely used for surface sterilization in plant cell and tissue culture experiments [19, 20]. The high concentration of sodium hypochlorite stimulates seeds germination that could be due to scarification effect on the seed coat that allows improving the permeability for water and oxygen or due to the improvement of oxidative respiration by producing more oxygen through the decomposition of sodium hypochlorite [21].

Table 2-The effect of sodium hypochlorite and sterilization time on the percentage of *P. harmala* seeds germination after 20 days.

Sodium hypochlorite **%**	**Sterilization** time **(minutes)**			**Mean**
	5	**10**	**15**	
1.5	76.7	66.7	70.0	71.1 a
3	50.0	70.0	60.0	60.0 c
4.5	70.0	66.7	60.0	65.6 b
6	90.0	70.0	56.7	72.2 a
LSD		7.43		4.29
mean	71.7 a	68.3 a	61.7 b	
LSD		3.71		

*each value in the table is the average of three replicates

The control medium which was not supplemented with any plant growth regulators gave no results for callus induction this insurance the crucial role of phytohormones to stimulate callus in plant tissue culture process. Ten different media formulations were used for callus induction. The results showed that the explants respond differently to the different callus induction media as shown in Table -3 and Figure-2. The highest percentage of callus induction 54% was noticed in stem explant followed by root and cotyledons with 48% and 46% respectively. While the true leaves were given a lower percentage of callus induction 28%.

Among all callus induction media, MS_8 was the most responsive medium with an average 90% of callus induction and selected as maintenance media. Conversely, MS_5 record lowest callus induction percent 10%.

The interaction percent appears in root and stem which explanted on MS_8 medium that gave 100% induction altogether, on the other hand, the lower percentage was noticed for all explants in MS_5 where the BA and 2, 4-D were not working synergistically for induction as a combination in 0.5 mg/l concentration for each one. In the case of the cotyledon leaf, it was induced by MS8 and MS9 to give 90 and 80% respectively. The cotyledon on 0.5 mg/l from NAA lonely with a percentage 80% raised the average of MS_9 to 60% which also considerably better than the 2, 4-D lonely in MS_{10} which gave 40%. NAA Auxin may be work lonely as in MS_9 but the BA Cytokinin perhaps was weakens its work as seen in MS_1.

The selected medium was found very much efficient and after four to six weeks all the explants were fully covered 100% on MS_8 media with compact and friable in texture, granular and white to light greenish in color mass of undifferentiated cells of callus (Figure-3). The initiation of the callus was perhaps due to the exogenous supply of growth regulators which disturbed the established polarity and induced the callus formation [12]. The type and concentration of plant growth regulators and the type of explants have a great influence on callus induction [22].

The deficiency of callus formation in some of medium in this study refers to the limited level of endogenous hormones in *P. harmala* toward callus formation. This clue that a plant growth regulator is a key factor responsible for callus initiation and development in plant cells.

The results in Table-3 indicated that the combination of 2, 4-D and kinetin was effective to satisfactorily induce callus from the root and stem explants of *P. harmala*. As shown in the result that kinetin (a type of cytokinin) in 2 mg/l concentration in conjunction with the presence of 2,4-D (a type of auxin) gave a high percentage to callus induction the two are complementary because Kinetin often used in plant tissue culture for promoting cell division [23] and the auxins have a cardinal role in coordination of many growth and behavioral processes in the plant's life cycle and are essential for plant body development. In addition to auxin wounding has been reported to promote callus formation in many plant species [24]. 2, 4-D has an ability to revert cells in the explant to dedifferentiated state and begin to divide rapidly [25]. In addition, [26] showed that under optimized culture conditions the high rate of callus induction and proliferation in *Acacia confusa Merr.* immature leaflet explant was

obtained after 35 days on MS medium supplemented with 3 mg/l 2,4-D, 0.01 mg/l NAA and 0.05 mg/l Kin.

Table 3- The effect of media and explants on callus induction % of P. *harmala* after 4 weeks

Media type	Explants %				mean
	root	stem	cotyledon	True leaf	
Cont.	00.0	00.0	00.0	00.0	00.0
MS_1	50.0	60.0	60.0	30.0	50.0
MS_2	40.0	20.0	60.0	10.0	32.0
MS_3	50.0	70.0	40.0	30.0	47.5
MS_4	50.0	40.0	60.0	20.0	42.5
MS_5	20.0	20.0	00.0	00.0	10.0
MS_6	30.0	60.0	20.0	20.0	32.5
MS_7	40.0	30.0	10.0	20.0	25.0
MS_8	100	100	90.0	70.0	90.0
MS_9	60.0	70.0	80.0	30.0	60.0
MS_{10}	40.0	70.0	40.0	50.0	50.0
LSD	N.S				6.38
mean	48.0	54.0	46.0	28.0	
LSD	4.04				

*each value in the table is the average of ten replicates.

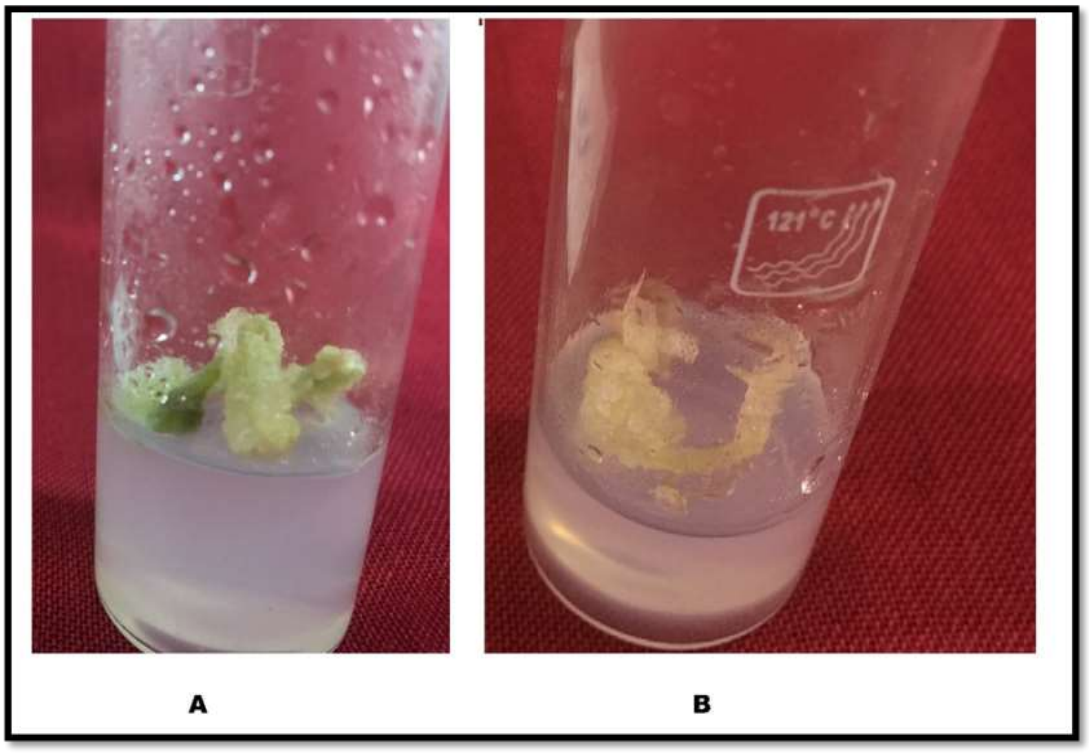

Figure 2-A: stem explant for callus induction on MS_1 media, B: root explant for callus induction on MS_3 media

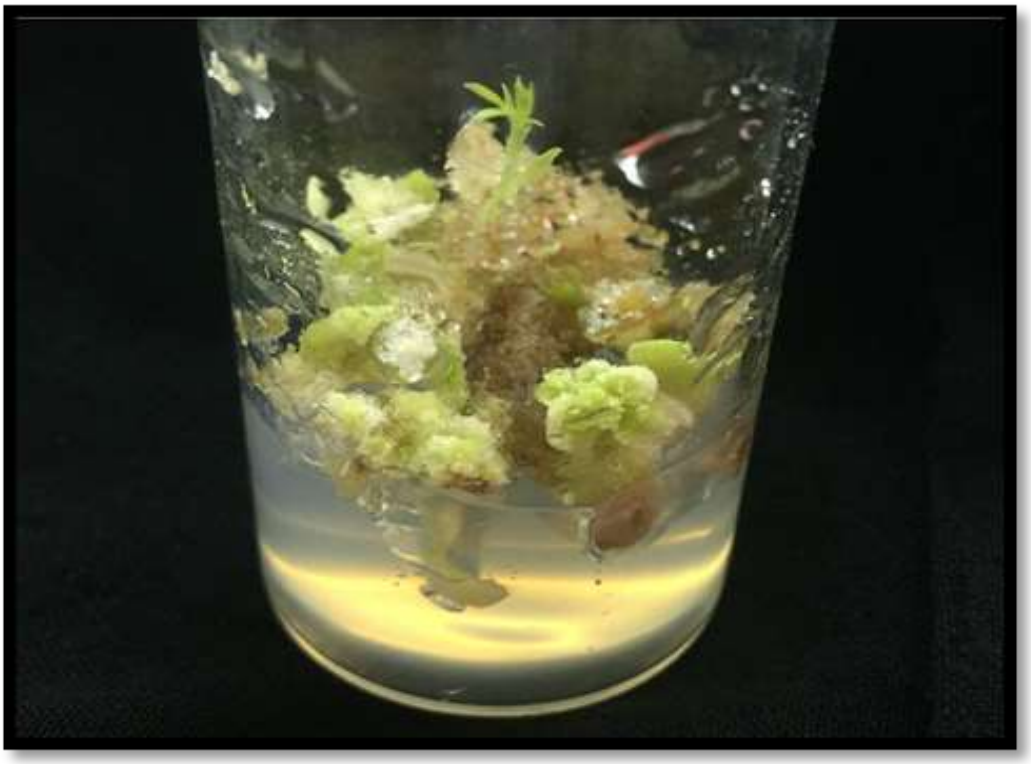

Figure 3-Granular, greenish in color mass of undifferentiated cells of callus from stem on MS_8 media after 5 weeks.

The results showed in Table-4 that MS_8 medium produced the highest amount of callus fresh weight with mean 1276.3 mg which was significantly different with other medium combinations followed by MS_6 with mean 795.9 mg fresh weight, and the lower fresh weight was recorded on MS5 in 377.8 mg. The results showed that there were no significant differences between MS_1 and MS_3 with average 515.3 mg and 493.1 mg respectively, also between MS_5 and MS_9 with approximate means 377.8 mg and 498.3 mg respectively.

The obtained results in term of explants effect indicated that the maximum average amount of the root explants produced callus fresh weight was 734.6 mg that the maximum amount of callus fresh weight with an average of 734.6 mg when cultured on MS_8 medium followed by the stem explants which gave an average of 653.6 mg of callus fresh weight and cotyledon leaves explants with 492.9 mg.

In the other hand, the true leaf explants were excluded from this analysis and recorded negative results because of the most of them gave a very few amounts or no induction of callus on the cut edge for almost medium used. The interaction analysis between the media and the explants showed that the root explants grown on MS_8 gave the highest callus fresh weight 1954.0 mg followed by stem explants in a value 1170.0 mg, while the lowest callus fresh weight was for cotyledon leaf explants 00.00 mg grown on MS_5 which was not give any induction of callus. The result of the current experiment indicated that callus induction and propagation from *P. harmala* are affected by the growth regulators combination and explants as with other plants and researches.

The hypocotyl and cotyledon explants of *P. harmala* on MS medium supplemented with 1 mg/l and 2 mg/l 2, 4-D was efficient for callus initiation [27]. Whereas [13] found that the highest callus induction from *Brassica nigra* L. stem explant was obtained on MS_2 medium supplemented with 2, 4-D and Kin also the highest fresh weight was obtained from stem explant in the present of BA, 2, 4-D and NAA.

Table 4-The effect of plant hormones on fresh weights of callus of *P. harmala* after four weeks

Media type	Fresh weights (mg)			Media
	root	stem	cotyledon	Media Mean
Cont.	00.00	00.00	00.00	00.00g
MS1	621.0	521.0	413.0	518.3 e
MS2	501.0	705.0	685.0	630.3 c
MS3	735.0	387.0	357.3	493.1 e
MS4	681.3	490.0	630.3	600.6 cd
MS5	739.0	394.3	00.0	377.8 f
MS6	624.0	886.0	877.0	795.7 b
MS7	520.0	814.0	482.0	605.3 cd
MS8	1954.0	1170.0	705.0	1276.3 a
MS9	481.0	255.0	459.0	398.3 f
MS10	490.0	913.7	320.0	574.6 d
LSD	90.48			52.24
Mean	734.6 a	653.6 b	492.9 c	
LSD	28.61			

*each value in the table is the average of ten replicates

The results of Table-5 regarding with dry weight showed that MS_8 medium introduced a higher mean value in callus dry weight 57.07 mg compared with other media, but there was no significant difference with MS_7 which gave 42.20 mg dry weight. The table indicated that the stem and root explants were significantly different with cotyledon with an average of 39.9, 36.4 and 25.6 mg respectively.

Moreover, the interaction analysis between media and explants showed that the root explants grown on MS_8 gave the highest callus dry weight 74.60 mg followed by the stem in a value 60.30 mg on the same media whilst the lowest value was 00.00 mg for cotyledon grown on MS_5 which did not give any induction of callus.

As a result showed in both Tables-(4, 5) there was variance in callus fresh and dry weight may belong to the humidity of fresh callus that gave dissimilarity with dry weight also may be because the type of callus, especially in root which was friable and softy. Anyway, the dry weight considers as an indicator for research rather than fresh weight. [13] Found that MS_1 medium was significantly higher in callus dry weight 52.80 mg supplemented with 2.5 mg/l BA, 0.5 mg/l 2, 4-D and 0.5 mg/l NAA compared with other media. The dry weight affected by many factors such as plant growth regulators, type of explant, plant species also the culturing factors like dark and light [28].

Table 5-The effect of plant hormones on dry weights of callus of *P. harmala* after four weeks

Media type	Dry weights (mg)			Media
	root	stem	cotyledon	Media Mean
Cont.	00.00	00.00	00.00	00.00g
MS1	39.60	35.60	26.20	33.80 bcd
MS2	31.00	35.00	42.00	42.01 b
MS3	35.5	24.00	19.70	26.41 cde
MS4	34.00	39.00	31.30	34.77 bc
MS5	38.23	25.36	00.00	21.20 ef
MS6	29.46	50.00	40.00	39.89 b
MS7	37.70	57.60	31.30	42.20 a
MS8	74.60	60.30	36.30	57.07 a
MS9	22.16	14.10	14.30	16.81 f
MS10	21.60	40.5	14.50	25.46 de
LSD	14.95			8.63
Mean	36.39 a	39.95 a	25.55 b	
LSD	4.73			

The results in Table-6 showed the GC-analysis of Harmine concentration between the root and stem-induced callus on MS_8 medium and the root and stem in the field. As shown in the table that the field stem gave a high concentration of harmine reached to 56.13 µg followed by the field root with 40.95µg value, while stem-induced callus gave a low concentration of harmine record 03.69 µg also the root-induced callus with very low value 03.00 µg record. This may be related to the concentration of auxins higher than the normal state in field plant lead to decreasing in secondary metabolites production. Auxins in the presence of low levels of kinetin induce the rapid disorganization of transformed roots of *Nicotiana rustica* ultimately to form suspension cultures of transformed cells and this process is associated with a decrease in nicotine content of the cells. This is related to cells in the culture losing competence in alkaloid biosynthesis [29], the auxins in a high concentration such as 9 mg/ l from NAA may be because decreasing the secondary metabolites like anthraquinone, phenolic and flavonoid contents in *Morinda citrifolia* [30].

Table 6-he concentration of Harmine alkaloid in root and stem *in vivo* and *in vitro*

Samples	Harmine µg
Field root	40.95
Field stem	56.13
Root-induced callus	03.00
Stem-induced callus	03.69

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