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Plant germination and production of callus from the yellow hornpoppy (*Glaucium flavum*): the first stage of micropropagation

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The yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae) is a perennial herb, distributed in the Mediterranean region, including Egypt. The plant contains many benzyl isoquinoline alkaloids from the aporphine type such as glaucine, isoboldine, l-chelidonine, l-norchelidonine and 3-O-methylarterenol, making it to display various medicinal activities including antitussive, anticancer, antioxidant, antimicrobial, antiviral, hypoglycemic, analgesic, antipyretic, bronchodilator and anti-inflammatory effects. The plant is now rare and endangered in the Egyptian flora due to urban sprawl. The present study looks into *Glaucium flavum* seeds' *in vitro* germination as well as the ability of the explants taken from the growing seedlings to form stable callus lines in order to enable micropropagation as a way to save the rare plant. The study also scans the production of different medicinally valuable alkaloids, particularly glaucine, in produced callus.

1. Introduction

The yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae) is a perennial herb, up to 60 cm tall, with pale green leaves that are infolded along their margins. Its stems bear yellow poppy-like flowers in spring and summer and then these flowers give rise to elongated siliquiform capsules. The name 'horned-poppy' is derived from its very long, swollen and pointed capsules, sometimes bearing horn-like protrusions (Bercu et al. 2006). Yellow horned poppy is distributed in the Mediterranean regions and also found along the Atlantic cost of Europe, from Portugal and Spain to England, Ireland and Norway and also found along the Black Sea coast (Schreter 1976). This plant becomes a retreat in many parts of Europe (Solås et al. 2004) and it is subjected to be rare and endangered in the Egyptian flora due to urban sprawl especially along the Northern coast area.

The aerial part of the plant contains many isoquinoline alkaloids such as glaucine, isoboldine, l-chelidonine, l-norchelidonine, 3-O-methylarterenol, corunnine and sanguinarine (Schreter 1976). The plant exhibits various pharmacological properties including antitussive (Aleshinskaya 1976), anticancer (Bournine 2013; Hadjiakhoondi 2013), antioxidant, antimicrobial, antiviral (Spasova et al. 2008), hypoglycemic (Chi et al. 2006) analgesic, antipyretic (Pinto et al. 1998) and bronchodilator and anti-inflammatory (Cortijo et al. 1999) activities. The antitussive effect of glaucine has many advantages over the currently available antitussive preparations, especially codeine, including less depression activities on respiration, no inhibiting effect on intestinal motility and no habit-forming or addictive properties.

Plant *in vitro* propagation techniques have been used in the conservation of threatened plants in recent years and this trend is likely to continue as more species face risk of extinction (Kapai et al. 2010; Sarasan 2006). *In vitro* propagation offers an easy, rapid and space-efficient way for banking of plant species. The propagation occurs from seedlings or different viable parts of the rare and endangered plant species to investigate the possibility of regeneration of whole plantlets and transfer them to *ex vitro* conditions (Debnath et al. 2006; Nalawade and Tsay 2004; Wala and Jasrai 2003).

To the best knowledge of the authors there is no report to discuss the *in vitro* germination and callus induction of the yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae). The present study aims to investigate the tendency of the yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae) seeds to germinate *in vitro*. The ability of the explants taken from the growing seedling to form stable callus formation is determined representing the first step for micropropagation. The study also looks into the production of different medicinally valuable alkaloids, particularly glaucine, in produced callus.

2. Investigations, results and discussion

The yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae) has become a rare plant in Egyptian flora now due to many human activities such as overgrazing and urban sprawl. This situation inspired the authors to carry out this study which focused on *in vitro* seed germination and callus induction as a first step for the whole micropropagation for this plant.

2.1. Seed germination percentage

Glaucium flavum seeds were cultured on sterile Whatman grade number 1 filter papers in sterile petri dishes. Different concentrations of gibberellic acid (GA3) ranging from 20 to 150 mg/L

Table 1: Seed germination percentage of the yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae)

Treatment	Seeds germination percentage in days											
	2 days	4 days	6 days	8 days	10 days	15 days	20 days	25 days	30 days	40 days	50 days	60 days
No treatment (0)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	22.22	47.22	69.44
No treatment (4)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.78	16.67	52.78	66.67
No treatment (8)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.78	13.89	38.89	63.89
GA3 20 mg/l (0)	0.00	0.00	0.00	0.00	0.00	27.78	52.78	77.78	77.78	77.78	77.78	77.78
GA3 20 mg/l (4)	0.00	0.00	0.00	0.00	0.00	27.78	58.33	83.33	83.33	83.33	83.33	83.33
GA3 20 mg/l (8)	0.00	0.00	0.00	0.00	0.00	25.00	47.22	61.11	66.67	66.67	66.67	66.67
GA3 40 mg/l (0)	0.00	0.00	0.00	0.00	27.78	41.67	52.78	69.44	83.33	83.33	83.33	83.33
GA3 40 mg/l (4)	0.00	0.00	0.00	0.00	27.78	44.44	47.22	58.33	72.22	88.89	91.67	91.67
GA3 40 mg/l (8)	0.00	0.00	0.00	0.00	16.67	38.89	50.00	66.67	72.22	72.22	72.22	72.22
GA3 50 mg/l (0)	0.00	0.00	0.00	52.78	69.44	83.33	94.44	94.44	94.44	94.44	94.44	94.44
GA3 50 mg/l (4)	0.00	0.00	0.00	52.78	52.78	77.78	86.11	86.11	86.11	86.11	86.11	86.11
GA3 50 mg/l (8)	0.00	0.00	0.00	55.56	61.11	72.22	86.11	91.67	91.67	91.67	91.67	91.67
GA3 100 mg/l (0)	0.00	0.00	0.00	25.00	38.89	63.89	75.00	75.00	75.00	75.00	75.00	75.00
GA3 100 mg/l (4)	0.00	0.00	0.00	16.67	38.89	55.56	61.11	61.11	61.11	61.11	61.11	61.11
GA3 100 mg/l (8)	0.00	0.00	0.00	25.00	41.67	50.00	69.44	72.22	72.22	72.22	72.22	72.22
GA3 150 mg/l (0)	0.00	0.00	0.00	0.00	11.11	27.78	50.00	50.00	50.00	50.00	50.00	50.00
GA3 150 mg/l (4)	0.00	0.00	0.00	0.00	22.22	30.56	36.11	50.00	50.00	50.00	50.00	50.00
GA3 150 mg/l (8)	0.00	0.00	0.00	0.00	13.89	33.33	36.11	38.89	38.89	38.89	38.89	38.89

The seeds were allowed to germinate in dark at 15 °C growth room. GA3 concentration was shown in the treatment column followed by stratification days in brackets. Stratification was done by putting seeds in 4 °C. The experiments was repeated 3 times using 12 seed as an initial number of seeds (n = 3). The results show the mean of seed germination percentage and standard error of mean was not put for simplification and did not exceed 10 % of mean.

were tried in this experiment. Stratification of seeds was also investigated by putting seeds in 4 °C for zero, four and eight days before putting in a growth room. After stratification days, seeds were allowed to germinate in the dark at 15 °C in growth room. Seed germination percentage is shown in Table 1. The results show that if seeds were not treated with GA3, germination begins after 30 days with very low germination percentage (Table 1). However, when GA3 was added, seeds showed better germination percentage which reached its maximum when 50 mg/L concentration of GA3 was used. At this GA3 concentration, seeds begin to germinate after 8 days with high germination percentages up to 52 %. However, in higher concentrations of GA3, germination percentages began to decrease making 50 mg/L GA3 is the optimum concentration for seed germination. Stratification did not have any significant effect on germination percentage of the seeds as shown in Table 1. Investigation of the effect of temperature was done by growing seeds in 15 °C and 22 °C and the results are shown in Fig. 1. The results indicated that seed germination percentage in 15 °C was significantly higher than that in 22 °C. However, there was no change in the time of seed germination but the initial germination percentage fluctuated from nearly 53% at 15 °C to 8.5% in 22 °C. The maximum germination percentage reached to 94.5% after 20 days in 15 °C and 64% after 30 days in 22 °C.

Gibberellic acid (GA3) plays an important role in the germination process (Ritchie and Gilroy 1998) and it is one of the hormones known for its ability to break dormancy in seeds (Nadjafi et al. 2006; Vieira et al. 2002). External application of gibberellins to seeds can break seed dormancy and aid seedling establishment (Nadjafi et al. 2006). Cold stratification also plays an important role in providing the stimulus required to overcome dormancy. Cold stratification has been reported to induce an increase in GA3 concentration (Bourgoin and Simpson 2004; Nadjafi et al. 2006). This study is a good example of the effect of GA3 in seed dormancy breaking, however, stratification did not have any effect in the germination percentage.

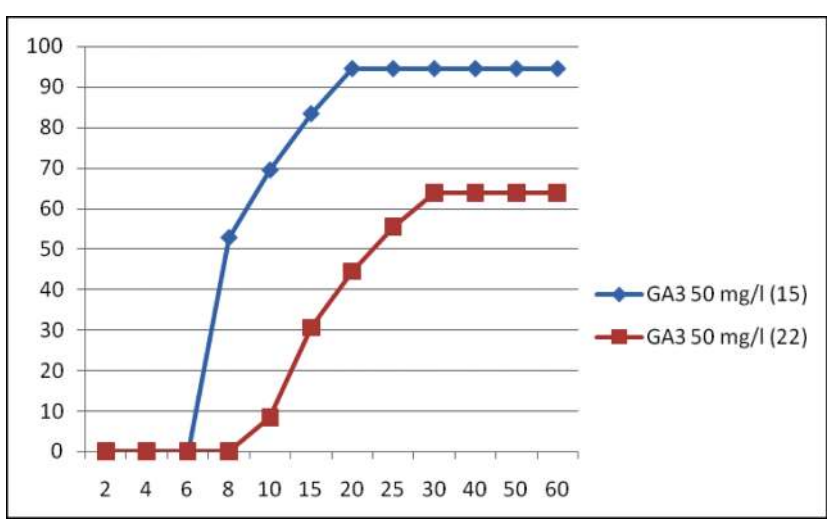


Fig. 1: Effect of temperature on seed germination percentage of the yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae). The results are presented as the mean of three independent samples (n = 3). Standard error of mean was not put for simplification and it did not exceed 10% of mean.

2.2. Seed germination rate

Seed germination rate was investigated at different concentrations of GA3 and under different stratification periods, Fig. 2. The results indicated that the best growth rate was achieved when 50 mg/l GA3 was used. The length of seedling reached almost 8 cm after nearly two months. The results show that the seed germination rate was slow relative to other plant seedlings. The seedlings were weak even when allowed to grow on MS solid medium. Stratification periods neither have any effect on the seed germination rate nor on the seedling health and viability.

2.3. Callus induction

The produced seedlings were used as explants for production of callus. Induction of callus was studied using different parameters such as the percentage of callus induction, days for callus induction and callus growth rate measured through callus weight and callus diameter. Different phytohormones and phytohormone-combinations were used in this study and the successful attempts in callus production are listed in Table 2. Generally, callus production was challenging and callus initiation time was long relative to other plants. The rate of callus growth was slow and the callus showed high production of phenolic compounds resulting in browning. One of the best hormonal combinations used was NAA and BAP (1:0.5 mg/L) which was able to induce all explants to give callus (100% callus induction percentage, Table 2) and the callus was produced in an average of 32 days. The callus weight reached to 0.85 g within 60 days with a diameter of 7.3 mm indicating high callus growth rate relative to other experiments. However, this combination gave brown callus after 60 days and several attempts for subculture was not successful to decrease the coloration nevertheless the growth rate did not change. Many methods were used to control the browning such as using charcoal, ascorbic and citric acids. The use of sterile ascorbic acid solution (15 mg/l) slightly improved the case however this did not improve or affect the rate of growth. Another phytohormone combination which gave good callus production was BAP, 2,4-D and TDZ (0.1:1:0.5 mg/L). This combination showed callus induction percentage of 75% with relatively good callus initiation days' value of 30 days. The combination gave the best callus growth rate represented by relatively high callus weight and diameters as shown in Table 2. The callus colour was brownish white showing less phenolic compound and less browning as shown in Fig. 3. All successful attempts for callus production were carried out in dark at 15 °C. Increasing temperature to 22 °C failed to produce callus in all cases. Media other than MS such as LS and Gamborg B5 were also tried in callus induction and production experiments without showing any significant changes.

2.4. HPLC analysis

Produced calli were investigated for the production glaucine, the well known benzyl isoquinoline alkaloid and the main constituent to which the medicinal values of the yellow hornpoppy *Glaucium flavum* are attributed. The calli were extracted and scanned for the production of such a valuable alkaloid using HPLC. Glaucine was isolated from the wild plant and its structure was elucidated using different spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, two-dimensional NMR and mass spectrometry. The isolated glaucine was used as an authentic compound in this analysis. Figure 4A shows the response injection of authentic glaucine, a signal appeared at 9.3 min which was used to make a calibration curve of the alkaloid, Fig. 4B. The curve was linear between the concentrations 100 μg/ml to 10 μg/ml with a lower detection limit at 100 ng/ml. Scanning of

Table 2: Callus induction of the yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae)

Code	Treatment						Percentage of callus induction	Days for Callus initiation	Callus weight mg in days				Callus diameter mm in days				Callus colour after 60 days
	NAA	BAP	2,4D	Kin	TDZ	Temp			15	30	45	60	15	30	45	60	
NB10115	1	0.1	–	–	–	15	66.67	30.33	0.00	200.00	383.33	526.67	0.00	2.33	4.00	6.00	Black
NB10515	1	0.5	–	–	–	15	100.00	32.67	0.00	110.00	510.00	843.33	0.00	2.67	5.00	7.33	Brown
NB20515	2	0.5	–	–	–	15	16.67	42.67	0.00	0.00	233.33	1036.67	0.00	0.00	3.33	10.33	Creamy white
NB30515	3	0.5	–	–	–	15	66.67	45.00	0.00	0.00	260.00	766.67	0.00	0.00	3.00	7.33	yellowish red
NB50515	5	0.5	–	–	–	15	95.83	43.67	0.00	0.00	148.33	676.67	0.00	0.00	2.00	6.33	Yellowish red
NB70515	7	0.5	–	–	–	15	91.67	45.67	0.00	0.00	141.67	586.67	0.00	0.00	2.00	4.67	Yellowish red
NB10522	1	0.5	–	–	–	22	89.64	60.00	0.00	0.00	0.00	166.67	0.00	0.00	0.00	2.00	Yellowish red
BD20115	–	0.1	2	–	–	15	33.33	42.00	0.00	0.00	116.67	250.00	0.00	0.00	2.00	4.00	Brownish black
BD40315	–	0.3	4	–	–	15	20.83	44.67	0.00	0.00	93.33	233.33	0.00	0.00	2.00	4.00	Brownish black
TDB0510115	–	0.1	1	–	0.5	15	75.00	30.33	0.00	206.67	1110.00	2156.67	0.00	2.33	10.67	25.33	Brownish white
TDB0120115	–	0.1	2	–	0.1	15	58.33	32.67	0.00	163.33	776.67	1420.00	0.00	2.00	7.67	15.00	Brown
TDB0520115	–	0.1	2	–	0.5	15	75.00	28.33	0.00	166.67	490.00	730.00	0.00	2.00	4.33	6.67	Reddish brown
DK050115	–	–	0.5	0.1	–	15	66.67	33.67	0.00	0.00	110.00	310.00	0.00	0.00	3.00	5.00	Brown
DK10115	–	–	1	0.1	–	15	54.17	42.67	0.00	0.00	103.33	280.00	0.00	0.00	3.00	5.00	Brown

Explants were taken from plant seedling growing under sterile conditions The experiments was repeated 3 times using 12 explants. The results show the mean and standard error of mean was not put for simplification and did not exceed 10 % of mean.

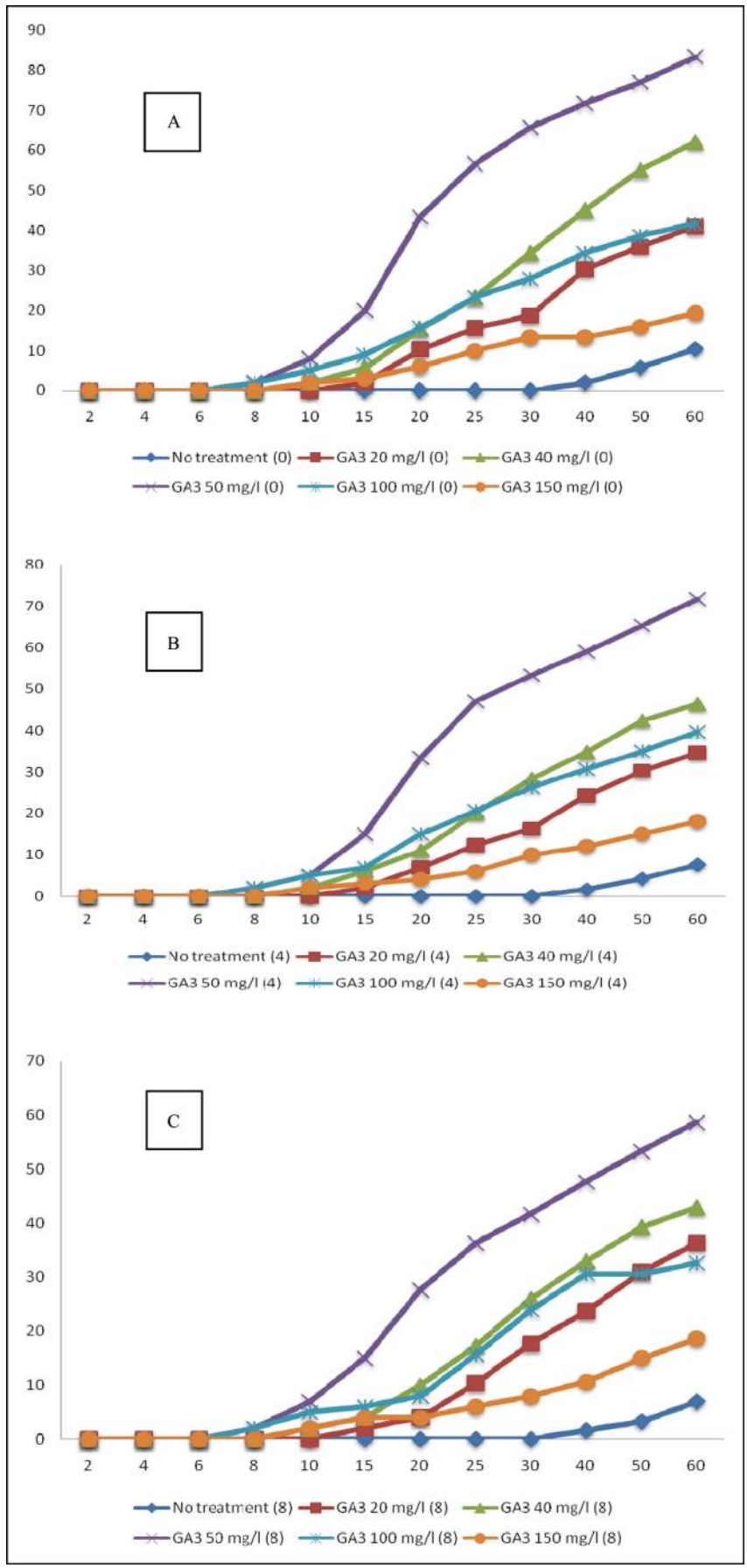


Fig. 2: Seed germination rate of the yellow hornpoppy, *Glaucium flavum* Cr. (Fam. Papaveraceae). The seeds were allowed to germinate in dark at 15 °C in growth room. GA3 concentration was shown in the treatment column followed by stratification days in brackets. Stratification was done by putting seeds in 4 °C. a: no stratification. b: stratification was allowed for four days. c: stratification was allowed for eight days. The experiments was repeated 3 times using 12 seed as an initial number of seeds (n = 3). Seed germination rate is represented by the total length of the seedling in mm in different time intervals. The results show the mean of seed germination rate and standard error of mean was not put for simplification and did not exceed 10 % of mean.

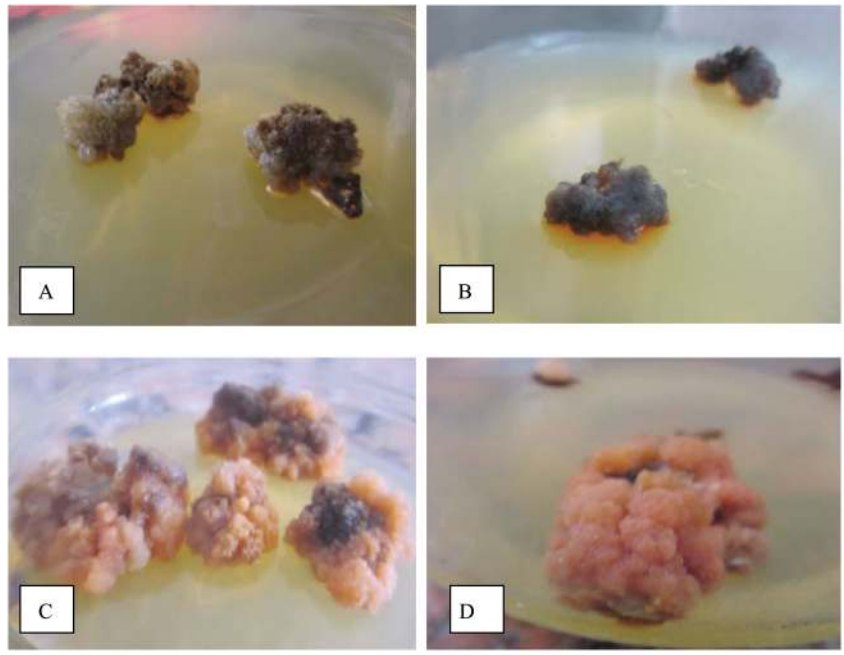


Fig. 3: Callus production from yellow hornpoppy *Glaucium flavum* Cr. (Fam. Papaveraceae); hormonal combination were as follow: a and b: 1.0 mg/l NAA + 0.5 mg/l BAP; c, d: 0.5 mg/l TDZ + 1.0 mg/l 2,4-D + 0.1 mg/l BAP + 15 mg/l Ascorbic acid;.

the best callus in growth rate (Code: TDB0510115, BAP, 2,4-D and TDZ (0.1:1:0.5 mg/L), Table 2) for production of glaucine revealed that the alkaloid was produced, Fig. 4C and the production of the alkaloid was nearly 0.1 % w/w. Produced glaucine from the callus was confirmed through comparing retention time with the authentic sample and through mass spectrometric analysis (molecular ion signal at m/z 355 with typical fragmentation pattern to the authentic). This production rate is not even close to the percentage of glaucine production in wild plant which can reach 3.6 % (Peled et al. 1988), however, the production of this valuable aporphine alkaloid in the callus shows that the machinery of its production was neither affected by the tissue culture processing nor by the phytohormones combination. This can give the green light for proceeding towards micropropagation.

Further work could concentrate on the optimization of callus production as well as the production of higher concentrations of glaucine and other valuable alkaloids from callus. Trails on callus should be moved to cell suspension cultures, which will offer the ability for more conditions optimization, collecting the produced alkaloids from medium and may give a solution for the problem of callus browning. Trials for micropropagation through organogenisis or somatic embryogenesis should also take place.

The yellow hornpoppy, *Glaucium flavum* contains many valuable isoquinoline alkaloids, especially glaucine. In this study, conditions for plant germination and callus production were investigated. The best condition for seed germination was in 15 °C using 50 mg/l of GA3 to break seeds' dormancy. The best conditions for callus induction was using MS media at 15 °C and using NAA and BAP (1:0.5 mg/L) or BAP, 2,4-D and TDZ (0.1:1:0.5 mg/L) as phytohormone combinations. The produced callus suffered from browning which could be partially overcome using ascorbic acid (15 mg/l). Callus produced the valuable aporphine alkaloid glaucine, however the produced alkaloid's concentration was very low relative to wild plant production.

3. Experimental

3.1. Plant seeds' collection

The whole plant and seeds of *Glaucium flavum* Cr. (Family Papaveraceae) were collected on November 2010 from Sidi Barrani, North coast, Egypt. The plant was identified by Dr Sameeh I. Eldahmy, professor of pharmacognosy, faculty of pharmacy, Zagazig University. Voucher specimens were put in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University. Full grown seeds were obtained from the wild plant.

3.2. Seeds sterilization

Seeds were sterilized by mixing with 70 % ethyl alcohol for 1 min and then shaking with 50% commercial hypochlorite solution - 2 drops of Tween 20

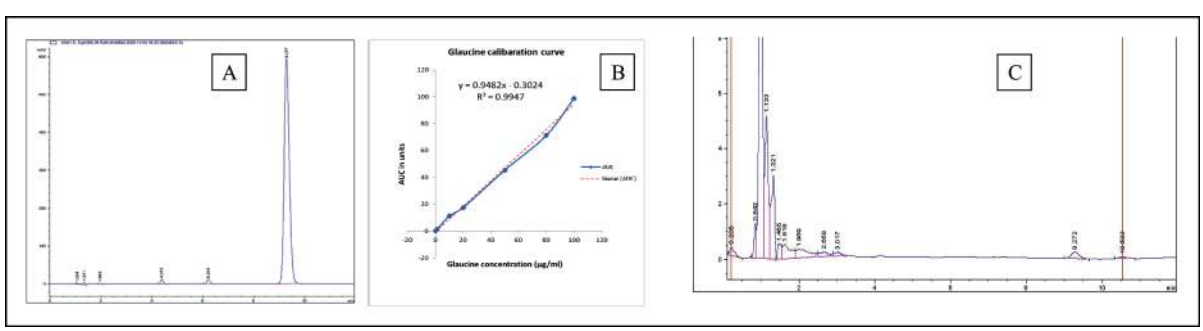


Fig. 4: High performance liquid chromatographic analysis of callus produced from the yellow hornpoppy, *Glaucium flavum*. a: HPLC chromatogram of authentic glaucine. b: Scatter analysis and calibration curve of glaucine. c: HPLC chromatogram of callus extract. Each authentic glaucine concentration in the calibration curve and callus quantification was repeated 6 times (n = 6).

solution for 20 min. Under the hood, the sterilized seeds were rinsed with sterile distilled water three times before applying to media for germination.

3.3. Germination of seeds

The sterilized seeds were cultured on sterile Whatman grade number 1 filter papers in sterile petri dish devoted from any growth regulator except of different concentrations of gibberellic acid (GA3). Petri dishes were placed in the refrigerator at 4 °C for a number of days (n) (n = 0, 4, 8 days) to allow stratification of seeds. After stratification days, seeds were allowed to germinate in the dark at 15 °C in the growth room. Germination of seeds was investigated using seed germination percentage and seedling germination rate parameters. Seed germination percentage was calculated using the following formula:

$$\text{Seed germination percentage} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds cultured}} \times 100$$

Seedling germination rate is represented by the total length of the seedling in mm in different time intervals. Twelve seeds were used for each experiment with three replicates (n = 3).

3.4. Production of callus

Seedlings were cut aseptically into small pieces (2–4 mm length) and used as a source of explants. Explants were cultured on solid MS medium supplemented with different growth regulators including naphthalene acetic acid (NAA), benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (K) and thidiazuron (TDZ) in different combinations and concentrations for initiation of callus. Agar (0.7%) was added for the solidification of the medium and the pH was adjusted to 5.8. Each treatment consisted of two explants per jar with four replicates. Cultured jars were maintained in growth room at 15 ± 2 °C. Callus induction and maintenance was investigated using three parameters: callus induction time, callus induction percentage and callus growth rate. Callus induction time is the number of days passed until the callus is formed and reach 2 mm in diameter. Callus induction percentage is calculated using the following formulae:

$$\text{Callus induction percentage} = \frac{\text{Number of callus produced}}{\text{Total number of explants cultured}} \times 100$$

Callus growth rate is represented by the total fresh weight (mg) and diameter (mm) of callus in different time intervals.

3.5. Analysis of alkaloids produced by callus

The callus was ground to fine powder and extracted by maceration with ethyl alcohol 75% five successive times and a sixth time with methylene chloride until the extract stopped to give precipitate with Mayer's reagent. The extract was concentrated under reduced pressure to obtain the total extract of callus. The callus extracts were defatted by shaking with hexane and the remaining extract was dissolved in the mobile phases and checked using HPLC.
The HPLC analyses were performed according to Bogdanov et al. (2012), on an Agilent 1100 series liquid chromatography system, equipped with a LC 1100 HPLC pump, a LC 1100 UV/Vis photodiode array detector tuned to 280 nm, an injector with a 20 µL loop and chemStation software for data treatment. An Eclipse C18 (150 x 4.6 mm i.d., 120 Å) was used as an analytical column. The mobile phase was a mixture of 0.1% triethylamine aqueous solution and acetonitrile (60:40) delivered at a flow rate of 1 mL/min.

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