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A Study of the Alkaloids in callusing plant tissues from a range of Turkish cultivars of *Papaver somniferum.*

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Abstract: Plant tissue cultures were obtained from the seeds of five varieties on the basis of petal colour) of *Papaver somniferum* and alkaloidal extracts were screend for the presence of secondary metabolites using thin layer chromatography and mass spectroscopy.

Two plant tissue culture media were used in this study, Murashige & Skoog and Gamborg B5, supplemented with 0.1 mg/l of kinetin in addition to one of either 1.0 or 5.0 mg/l of 2, 4-dichlorophenoxyacetic acid (2, 4-D). Seedlings growing on media with 1.0 mg/l 2, 4-D produced planlets, while those supplemented with the higher dose, 5.0 mg/l, produced calluses. Variations were found in cultures produced from different seeds and in these grown on different media containing different levels of plant-growth hormones.

Although all the original seed material contained unidentified non polar Dragendorff positive spots when examined using thin layer chromatography, none could be detected in either the calluses or plantlets produced from them. All five varieties of seed were grown on the four different media but only one, the callus derived from seed variety No. 1 and grown on a Murashige & Skoog medium supplemnetd with 0.1 mg/l kinetin and 5.0 mg/l 2, 4-D, contained one Dragendorff positive spot, which did not correspond to any of the standard alkaloids used, and was not identified by mass spectroscopy.

Türk Orijinli *Papaver somniferum* dan Doku Kültürü Yolu İle Elde Edilen Kalluslarda Bulunan Alkaloidler Üzerinde Bir Araştırma

Özet: Petal rengi farklı 5 değişik *Papaver somniferum* L. tohumlarından (Emiral/84, Papaver somniferum var anatolicum) doku kültürü ile kallus üretilmesi ve üretilen bu kalluslarda alkaloit sentezinin kontrolu üzerinde çalışıldı. Çalışma sırasında örneklerden alınan ekstrelerde sekonder metabolit olarak alkaloitlerin varlığı ince tabaka kromatografisi, ve kütle spektrostopisi kullanılarak tarandı.

Çalışma sırasında, 0.1 mg/l kinetin yanında iki değişik dozda (1.0 ve 5.0 mg/l) 2, 4-diklorofenoksiasetik asit (2, 4-D) ilavesi ile hazırlanmış Murashige & Skoog ve Gamborg B5 besi ortamları kullanıldı. 1.0 mg/l 2, 4-D hormon dozunda daha önceden çimlendirilerek her iki besi ortamına aktarılmış olan tohumlar sürgün verirken, 5.0 mg/l 2, 4-D hormon dozunda kallus oluşumu görüldü.

Yapılan kimyasal analizler sonucunda 5.0 mg/l 2, 4-D hormon dozunda ve Murashige & Skoog besi ortamında yetiştirilen 1 nolu varyeteden elde edilen kallusda Dragendorff ile pozitif leke elde edildi.

Introduction

Opium is the dried cytoplasm of a specialized internal secretory system, the laticifer. When the unripe capsule is cut, a cream-coloured latex oozes to the surface, where it dries to form a dark brown sticky material. Raw opium alkaloids have been identified in *Papaver somniferum* (1, 2), at least 25 of which occur in the latex (3). However, of prime importance from a medicinal viewpoint are the benzylisoquinolines, papaverine and noscapine (narcotine), and the phenanthrenenes (morphinans), codeine and morphine.

The opiates are industrial commodities of plant origin for which there is still considerable demand. The 1986 demand for legal opium is estimated to be in excess of 1.000.000 kg (4). The requirements of isolated alkaloids were 663.462 kg of codeine and 197.862 kg of morphine. Current supplies from legal cultivation of the opium poppy are adequate, although legal production and exportation has been limited since 1953 as a result of the United Nations Opium Conference Protocol. World requirements and the limited availability of codeine obtained directly from the poppy plant has made codeine

production through stable-cell cultures of the genus *Papaver* an obvious target for exploitation. *Papaver* species produce a wide range of isoquinolines, sometimes with very high yields, and within individual specise there is considerable intraspecific variation in alkaloid content (5). The major producers of the morphinans are *P. somniferum* L. and *P. bracteatum* Lindl., but this group of alkaloids has also been obtained in low yields from *P. fugax* L., *P. setigerum* D.C., *P. orientale* L., and *P. rhoeas* L. (5), and from herbarium material of *P. acrochaetum* Borm., *P. caucasicum* Bieb., *P. cylindricum* Cullen., *P. gracile* Boiss., and *P. persicum* Lind. (6).

Industrial production of opiates from tissue culture is dependent on the large accumulation of alkaloids in a cell-culture medium. While there has been great success in plant-cell culture in terms of cells with high yields of isoquinolines, from a commercial and pharmaceutrical viewpoint, the morphinans have proved difficult to produce in plant-cell cultures.

Most cultured *Papaver* cells, in the form of calluses or cell suspension, readily produce sanguinarine, dihydrosanguinarine, norsanguinarine, and oxysanguinarine (7, 8, 9). Isolations of magnoflorin (6), stylopine (10), cryptopine (11, 12), chelirubine (7), noscapine (13), protopine (14), orientalidine, and isothebaine (15) have also been reported.

Numerous reports of the productions of the morphinans thebaine, codeine, and morphine, from cell cultures of *P. somniferum* and *P. bracteatum* can be found in the literature, although yields are low compared with the high yields of the plants. Research suggests that culture conditions can be manipulated to promote morphinan alkaloid production (16).

The aim of this study was to find early-producing calluses with different media and hormone doses. The alkaloid production of the calluses was observed during various growth periods by analysing the callus extracts. The findings of this study should help enable the production of alkaloids of biological origin under controlled conditions. In other words, this is a starting point for the isolation of seconder metabolites in callus culture in Turkey.

Materials and Methods

In this study, seeds from *Emiral/84 and Papaver sommiferum var. anatolicum* L. of 4 different seed and petal colours were used (Table 1). All the seeds were surface sterilized with a 30% hydrogen-peroxide solution for two minutes, and germinated on wetted filter paper in Petri dishes in darkness at *ca* 25˚C. The 4-5-day-old seedlings were isolated and explanted on 2 different media. One of the media was Murashing and Skoog (17) and the other was Gamborg B5 (18). These two media contained supplements of 3% sucrose, 0.1 ppm, kinetin, and 1 ppm or 5 ppm 2, 4-D (dichlorophenoxyacetic acid). They were solidified with 1.2% agar end the pH was adjusted to 5.5. 250 ml flasks, each containing 50 ml of medium, were sterilized at 120˚C for 15 minutes before the addition of seedlings. Three samples of seedlings were prepared on each medium.

Alkaloidal control of seeds

1 g of each seed sample was weighed and extracted with 5 ml of chloroform: methanol (1:1) for 2 minutes. The solution was evaporated to 50 ml and 5 ml aliquots were chromatographed on Silica gel G/UV_{254} (0.25 mm).

Solvent system: Toluene-ethylacetate-diethylamine (70:20:10)

Detection reagent: Dragendorff reagent (19)

Seedling yields and alkaloidal control

Shoots were harvested and weighed to find their fresh yields, then dried at 40˚C overnight and weighed again (for dry yields). Alkaloidal extraction was carried out as described above.

Mass spectroscopy

Mass spectroscopic analysis was performed wit h a Kratos M-25 spectrometer fitted with a DS-55 cmoputer data output and with a DH88 mass spectroscopy data system. The mass spectra were determined at 70 eV. In

Sample	Seed Colour	Petal Colour	Types
1	grey-dark	red	Emiral/84
			Papaver somniferum
2	grey-dark	blue	var. anatolicum
3	white	white	"
4	grey	blue	"
5	pink	blue	"

Table 1. Characterization of the *Papaver somniferum* L. cultivars

the mass spectroscopic analysis of the samples, both electron impact (EI) and chemical ionizsation (CI) techniques were employed. Ammonia was the reagent used in the chemical ionisation process.

Results and Discussion

In this investigation two different plant tissue culture media were used, and in addition to 1.0 mg/l kinetin, two concentraitons of 2, 4-D (1.0-5.0 mg/l) were used to find out the effect of the plant growth hormones on the seedlings. According to the results, varieties 1 to 3 grew well on both media whereas varieties 4 and 5 did not grow (Picture 1) very well on either medium.

Seed varieties 1-3 grown on media containing 1.0 mg/l of 2.4-D produced planlets, and those grown on media containing 5.0 mg/l of 2.4-D, particularly variety 1, produced calluses (Picture 2).

Medium	Fresh yield (g)	Dry yield (g)	Moisture Content (%)
M&S (5.0 mg/l)			
1	0.8445	0.0229	97.2
2	0.9823	0.0747	92.4
3	0.8545	0.0515	93.97
4	0.9327	0.0707	92.42
5	0.3836	0.0149	96.12
M&S (1.0 mg/l)			
1	2.3948	0.1605	93.3
2	1.2363	0.0882	92.87
3	1.3626	0.1214	91.09
4	0.5423	0.0554	89.78
5	0.3918	0.0292	92.55
GB5 (5.0 mg/l)			
1	0.7263	0.0743	89.77
2	0.3663	0.0383	89.54
3	0.7301	0.0733	89.96
4	0.7063	0.0657	90.7
5	0.0802	0.0049	93.89
GB5 (1.0 mg/l)			
1	1.5002	0.1035	93.1
2	3.0156	0.1876	93.78
3	1.6336	0.1576	90.38
4	1.526	0.0998	93.46
5	-	-	-

Table 2. Level of fresh and dry yields of seedlings and their moisture contents (seedling yields/per culture flask).

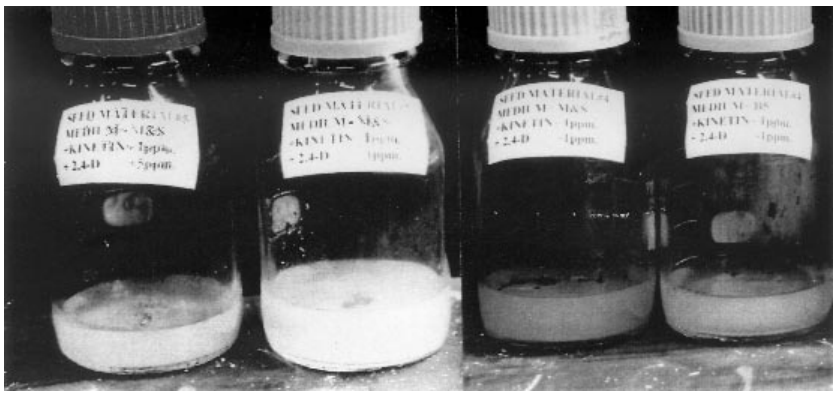

Figure 1. Appearence of varieties 4 and 5 with different media and hormone doses

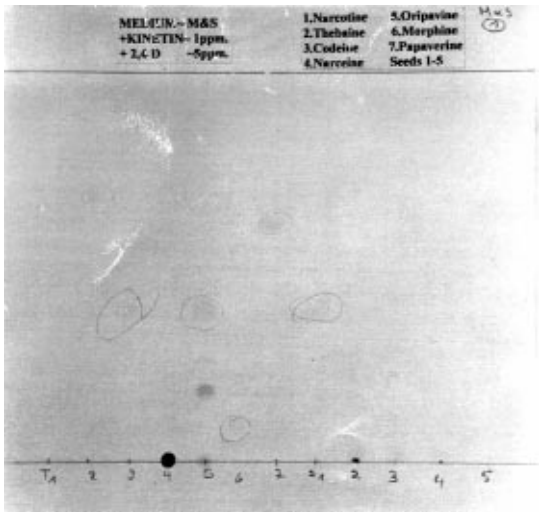

Figure 2. The effect of different levels of the plant growth hormone 2,4-D on the differentiation of the seedlings of variety 1.

The results in Table 2 show no correlation between moisture content and state of differentiation, but undifferentiated calluses would be expected to have a higher moisture content.

When examined using thin layer chromatography, the original seed material did not exhibit any Dragendorff positive spot corresponding to any of the reference alkaloids used as the standards.

All five varieties of seeds were grown on the four different media, but only callus derived from seed variety. No. 1 grown on the Murashige & Skoog medium supplemented with 5.0 mg/l 2, 4-D contained a Dragendorff positive spot (Picture 3). The extract containing this spot was subjected to mass spectroscopy, but no fragments of typical opium alkaloids were detected. The other callus calluses and plantlets did not exhibit any Dragendorff positive spots.

Rf values of the standards:

T_1=0.80 T_5=0.38

T_2=0.21 T_6=0.09

T_3=0.36 T_7=0.62

T_4=-- S_1=0.38 (Variety. 1)

Figure 3. TLC chromatography of the standard alkaloids and seedling extracts.

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