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Palm Pollen Extracts as Plant Growth Substances for Banana Tissue Culture

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Abstract: Pollen extracts of date palm were used in tissue culture medium for banana as growth substances in comparison with growth regulators. Pollen extracts contain auxin (IBA) and tryptophan (auxin precursor). Water pollen extract (WPE) contains higher concentration of indoles compared with ethanol pollen extract (EPE). Most of treated plants showed highly growth characteristics such as shoot number, shoot length, root number, root length, fresh weight and dry weight compared to either the cytokinin (benzyl adenine) and/or the auxin (indole butyric acid and naphthalene acetic acid). On the other hand, high concentration of pollen extracts concentration in banana tissue culture medium inhibited the growth. The electrophoretic profile of subunits of explants obtained from different treatments was similar to the profile of MS explants. In conclusion, natural extracts such as pollen extract of date palm provided to be an excellent economic resources as growth substances.

Key words: Pollen extract · Date palm · Plant growth substances · Banana · Tissue culture

INTRODUCTION

Pollen is a fine, powder-like material produced by flowering plants and gathered by bees. Pollens are the male reproductive cells of flowers [1]. The early Egyptians and ancient Chinese used pollen as a rejuvenating medicinal agent. It has been called a "fountain of youth". Pollen preparations are distributed worldwide for dietary purposes and as diet supplement by increasing the total dietary intake [2]. They contain concentrations of phytochemicals and nutrients and are rich in carotenoids, flavonoids and phytosterols [3]. Moreover, they are good source of protein, amino acids, vitamins, dietary fiber, fatty acids, enzymes, hormones and minerals [4]. There is a little information available on the endogenous hormones of pollen. In some species, the endogenous hormones were determined by bioassay but the rigorous analysis of hormones involving GC-MS or enzyme-linked immunosorbent assay (ELISA) has only been done in a few cases [5]. Growth substances have been detected in the pollen of apple, orchid, tobacco, haselnut, shrup, pine and corn [6].

Most of the current tissue culture methods are based on technologies developed over twenty years ago. The commercial use of plant tissue culture primarily involves the production of large number of plants with minimum input expenses. The main factors which ultimately influence the commercial propagation of plants *in vitro* are: the selection of plant species, the physical environment and the chemical media for *in vitro* culture. Research efforts have addressed growth regulating compounds and salt mixes as substitutes to the expensive chemical nutrient media with low cost natural extracts [7]. The present study was designed to examine pollen extracts of date palm as plant growth substances in banana tissue culture and compare them with synthetic growth regulators.

MATERIALS AND METHODS

Materials

Pollen grains: Pollen grains of Egyptian date palm (*Phoenix dactylifera* L., variety El-Hayani) were collected at the end of March from Shabramant, Giza, Egypt.

Chemicals: Benzyl adenine (BA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), tryptophan (Trp) and Murashige and Skoog (MS) basal medium were purchased from Sigma Chemical Co., USA. TLC plates were purchased from Merck, Germany.

Methods

Preparation of Palm Pollen Extracts: Two different extracts were prepared from pollen grains of date palm using the procedure reported by Nagai *et al*. [8] with some modifications as follows: To prepare water pollen extract

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(WPE), 0.1 g of pollen grains was mixed with 10.0 ml of distilled water (DW). After one hour, the mixture was sonicated by ultrasonic probe (frequency at 6 kHz) for 30 s then centrifuged at 5,000 rpm for 10 min with the temperature set at 20°C. The resulting supernatant was used as water pollen extract in all experiments. The same method was used for the extraction of pollen with ethanol. After extraction with ethanol (0.1 g pollen and 10.0 ml ethanol), the solvent was removed from the obtained extract by evaporation. The residue was redissolved in the same volume of DW (10.0 ml). The obtained solution (ethanol pollen extract, EPE) was used in all experiments.

Determination of IAA and its Derivatives: Indole acetic acid and its derivatives were determined in water and ethanol pollen extracts using the Salkowski method as described by Lindow *et al*. [9] as follows: One milliliter of each pollen extract was mixed with 2.0 ml of Salkowski reagent (2.0 ml of 0.05 *M* $FeCl_3$ + 100 ml of 5% perchloric acid) in a small glass test tube and incubated at room temperature for 30 min. The optical density of the solution was measured at 530 nm using Jenway 6300 spectrophotometer. The concentration was calculated from the standard curve prepared using serial concentrations of standard IAA solution.

Separation of Indole Compounds by TLC: IAA and other indole derivatives of water and ethanol pollen extracts were identified by thin layer chromatography (TLC) according to the method of Ehmann [10] as follows: Analysis of indole compounds by TLC was performed on silica gel-covered glass plates using PW solvent: propanol/water (8:2, v/v). Pollen extracts and standard solutions of tryptophan, indole acetic acid and indole butyric acid were spotted on TLC plate. The run was carried out in glass jar, covered with glass cover at room temperature. The chromatogram was developed by spraying with Vanurk reagent. Vanurk reagent was prepared by mixing one volume of Ehrlich reagent (1.0 g of *p*-dimethylaminobenzaldehyde dissolved in 50 ml HCl conc.) with three volumes of Salkowski reagent (2.03 g of $FeCl_3.6H_2O$ dissolved in 500 ml DW and 300 ml H_2SO_4 conc). After spraying, the chromatogram was heated in a 100°C oven for 5 min then removed from the oven and allowed to cool at room temperature. The R_f values of all spots were measured.

Tissue Culture Experiment

Plant Material: Small suckers of banana (*Musa* spp.) plants of about 50-70 cm in length were carefully cut from mother plants grown in field. The leaves and upper portion of the pseudo stem were removed. All roots were cut off at the corm and the soil was washed off. Any site suckers found on the corm were removed. The outer leaf sheaths of the pseudo stem were pealed off one at a time. This procedure was used until only one or two young leaf primordial remains. The shoot apex is excised by marking four incisions into the corm beneath the apex. Under a specific conditions, the excised apexes were soaked in 20% chlorox (5.25% sodium hypochlorite) for 20 min. a few drops of tween 20 emulsifier were included in the surface sterilization treatment to enhance spreading. The shoot tip explants were then rinsed 4 times for 5 min, each in sterile distilled water to remove all traces of chlorox and then sterilized apex was transferred to the culture media.

Culture Medium and Culture Conditions: The culture medium used for all experiments was based on MS medium as described by Murashige and Skoog [11]. To one liter of MS medium without plant growth regulators, 8 g of agar, 30 g of sucrose and different concentrations of each pollen extracts (40, 80, 120, 160, 200, 400 or 600 ppm) were added. The control was consisted of basal MS medium supplemented with either BA (1, 1.6, 2 or 3 mg kg^{-1}) for shoot multiplication stage and/or IBA (0.20, 0.32, 0.40 or 0.60 ppm) and NAA (0.18, 0.29, 0.37 or 0.55 ppm) for rooting stage. Medium was adjusted to pH 5.8 with KOH solution before adding the agar. All media were sterilized by autoclaving at 121°C and 101 kPa for 20 min. Cultures were incubated at 24±2°C and exposed for 16 h per day to an illumination of 15.7 μmol/m^2/s provided by daylight-type fluorescent lamps. In the propagation and rooting stages, growth characteristics (number of shoots, shoot length, number of roots, root length, fresh and dry weights) were determined. Proteins of explants resulted from mostly better treatmentswere subjected for electrophoretic analysis (SDS-PAGE).

Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis: Proteins of explants resulted from mostly better treatments in tissue culture were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [12].

Statistical Analysis: The results were analysed by an analysis of variance ($P<0.05$) and the means separated by Duncan's multiple range test. The results were processed by CoStat computer program (1986).

RESULTS AND DISSCUSION

Total Indole Identification: Indole compounds content was colorimetrically determined in both pollen extracts. The obtained results (Table 1) showed that water pollen extract contains higher concentration of indoles (10 mg/g pollen) compared with ethanol pollen extract (9 mg/g pollen).

Since both pollen extracts contained considerable amounts of indoles, it is important to identify auxin precursors (tryptophan and its derivatives) in pollen extracts by a simplest method (i.e. thin layer chromatography). The pollen extracts and indole standards [tryptophan (Trp), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA)] were subjected to TLC separation. After separation and visualization processes, spots were marked with a pencil. The results obtained of R_f value of each spot are given in Table 2. TLC separation of indole standards (Trp, IAA and IBA) revealed only one spot with R_f values of 0.72, 0.90 and 0.96, respectively. At the same time, both pollen extracts gave 4 spots with R_f values of 0.72, 0.78, 0.87 and 0.96. From the results in Table 2, it could be revealed that both pollen extracts contained the same compounds (indole derivatives); hence the R_f values of separated spots were much closed. Moreover, both pollen extracts contained tryptophan (auxin precursor) and indole-3-butyric acid, hence the R_f values of spots No. (I) and (IV) of each pollen extracts were much closed to the R_f values of pure standard tryptophan spot and indole-3-butyric acid. In addition, TLC revealed that both pollen extracts did not contain IAA. According to Kaldewey [13], bands II and III may be kynurenine and anthranilic acid.

Application of Palm Pollen Extracts in Tissue Culture: Because pollens contain plant growth substances, including cytokinin (as mentioned in Introduction part); IBA; auxin precursor (Trp); and indole derivatives (data of the present results), the application of pollen extracts in tissue culture medium was carried out on Grand Nain banana. Palm pollen extracts were used instead of cytokinin (BA) in shoot multiplication stage or auxin (IBA and NAA) in rooting stage. There seems to be good scope for substituting the expensive plant hormones (BA and auxins) with low cost, easily prepared, natural extracts (i.e. palm pollen extracts). In addition, this is the first time for using pollen extracts as a source of plant growth substances in culture media.

Table 1: Total indole contents of pollen extracts (mg/g pollen)

Pollen extract	Indole content
Water extract	10±0.34
Ethanol extract	9±0.21

Values are means of three replicates ± SE

Table 2: R_f values of indole derivatives of pollen extracts separated by thin layer chromatography

Extract	Spot No.	R_f	Identification
WPE	I	0.72	Tryptophan
	II	0.78	Unknown
	III	0.87	Unknown
	IV	0.96	Indole-3-butyric acid
EPE	I	0.72	Tryptophan
	II	0.78	Unknown
	III	0.87	Unknown
	IV	0.96	Indole-3-butyric acid

Shoot Multiplication Stage: The results of shoot proliferation and elongation response to different concentrations of BA as control, water pollen extract or ethanol pollen extract are presented in Table 3. The present data revealed that pollen extracts succeeded as plant growth substances instead of cytokinin at shoot multiplication stage of banana tissue culture. The shoot number and shoot length response to water pollen extract were higher than to ethanol pollen extract while other growth characteristics of shoots were stronger using ethanol pollen extract than to water pollen extract. There was many differences in growth characteristics of shoots in response to different concentrations of pollen extracts, but, in general, it could be concluded that 200 ppm of water pollen extract improved most shoot-growth characteristics (shoot number, shoot length, fresh weight and dry weight) almost similar to 2 ppm of BA. On the other hand, the same concentration of ethanol pollen extract induced less efficiency.

Rooting Stage: Data in Table 4 show the effect of replacing auxins (IBA+NAA) by water pollen extract and ethanol pollen extract in tissue culture medium of Grand Nain banana on growth characteristics. As shown previously, many differences in growth characteristics of roots in response to different concentrations of pollen extracts were reported. Data revealed that the concentration of each pollen extract (WPE or EPE) for the good values of most growth characteristics of roots is 160 ppm. At this concentration, some growth characteristics of roots response to ethanol pollen extract were higher than to water pollen extract (root number and root length) whilst reversed results was observed with fresh weight of roots. The same value of root-dry weight was achieved

Table 3: Effect of replacing cytokinin (BA) by water pollen extract (WPE) and ethanol pollen extract (EPE) in tissue culture medium of Grand Nain banana on growth characteristics of shoots

Treatment	Conc.* (ppm)	Shoot No.	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	F/D
BA	1	3.33bcd	3.16f	2.71b	0.49efgh	5.53
	±0.19	±0.09	±0.06	±0.01		
	1.6	3.66abcd	3.58ef	1.13fg	0.39fgh	2.89
	±0.19	±0.05	±0.07	±0.03		
	2	4.49ab	7.08bc	2.44b	1.08a	2.25
	±0.47	±0.05	±0.11	±0.01		
	3	3.83abcd	3.91ef	3.37a	0.82bcd	4.10
	±0.28	±0.05	±0.33	±0.03		
WPE	40	3.66abcd	8.33b	2.19bc	0.84bc	2.60
	±0.33	±0.66	±0.36	±0.16		
	80	3.33bcd	7.33bc	1.68cdef	0.62cdef	2.70
	±0.33	±0.88	±0.30	±0.13		
	120	3.00cd	4.00ef	1.25defg	0.42fgh	2.97
	±0.00	±0.99	±0.11	±0.07		
	160	3.00cd	7.33bc	1.41defg	0.56efg	2.51
	±0.57	±0.33	±0.07	±0.08		
	200	4.66a	10.8a	1.75cde	0.56efg	3.12
	±0.33	±0.44	±0.26	±0.09		
	400	4.33ab	8.66b	1.52defg	0.46efgh	3.30
	±0.33	±0.33	±0.23	±0.10		
	600	3.66abcd	7.16bc	1.14efg	0.34gh	3.35
	±0.33	±0.44	±0.15	±0.07		
EPE	40	4.33ab	8.66b	3.58a	0.91ab	3.93
	±0.33	±0.33	±0.24	±0.09		
	80	4.00abc	8.00b	2.12bc	0.67cde	3.16
	±0.57	±0.57	±0.09	±0.03		
	120	2.83cd	6.00cd	1.78cd	0.46efgh	3.86
	±0.16	±0.99	±0.08	±0.03		
	160	3.66abcd	8.00b	2.41b	1.04ab	2.31
	±0.16	±0.00	±0.13	±0.03		
	200	3.33bcd	8.16b	1.25defg	0.59def	2.12
	±0.33	±0.60	±0.01	±0.02		
	400	3.00cd	5.83cd	1.10fg	0.34gh	3.23
	±0.57	±0.16	±0.01	±0.01		
	600	2.66d	4.83de	0.95g	0.30h	3.16
	±0.33	±0.44	±0.03	±0.02		
L.S.D	-	1.032	1.492	0.54	0.217	-

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$. F/D: fresh weight/dry weight. *Concentration as pollen grains equivalent

with both pollen extracts. Accordingly, the ratio of fresh weight to dry weight response to water pollen extract was higher than to ethanol pollen extract. In general, it could be concluded that 160 ppm of pollen possess, respectively, the activity of IBA (0.40 ppm)+NAA (0.37 ppm) in tissue culture medium at rooting stage.

In conclusion, results obtained indicate that using pollen extracts in the culture medium enhances growth of banana-shoot explants when compared to BA and enhances growth of banana-root explants when compared to IBA+NAA. It could be explained these results by the presence of cytokinin and indoles. The inhibitory effect of

Table 4: Effect of replacing auxins (IBA+NAA) by water pollen extract (WPE) and ethanol pollen extract (EPE) in tissue culture medium of Grand Nain banana on growth characteristics of roots ???

Treatment	Conc.* (ppm)	Shoot No.	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	F/D
IBA+NAA	0.20+0.18	16.16d	4.16gh	2.25def	0.98ab	2.29
	±0.28	±0.47	±0.07	±0.05		
	0.32+0.29	12.99fg	4.33fgh	1.99efgh	0.89abc	2.23
	±0.38	±0.00	±0.08	±0.01		
	0.40+0.37	16.66d	5.83de	3.06bc	0.46fg	6.65
	±0.19	±0.28	±0.39	±0.03		
	0.60+0.55	21.99b	6.49bcd	2.07efg	0.84bcd	2.46
	±0.38	±0.09	±0.02	±0.01		
WPE	40	23.66a	7.66a	4.06a	0.91ab	4.46
	±0.33	±0.33	±0.28	±0.15		
	80	22.33b	7.00abc	2.50cde	0.70cde	3.57
	±0.87	±0.00	±0.20	±0.05		
	120	12.00g	4.33fgh	1.76fghi	0.52efg	3.38
	±0.57	±0.33	±0.40	±0.07		
	160	18.33c	5.50def	3.57ab	1.04ab	3.43
	±0.33	±0.76	±0.26	±0.05		
	200	15.66de	5.33defg	1.55ghij	0.65def	2.38
	±0.33	±0.33	±0.09	±0.11		
	400	4.33h	2.83i	1.39hij	0.52efg	2.67
	±0.33	±0.16	±0.14	±0.05		
	600	3.66h	2.66i	1.02j	0.41g	2.48
	±0.33	±0.33	±0.05	±0.04		
EPE	40	24.33a	8.00a	3.82a	0.85abcd	4.49
	±0.33	±0.57	±0.15	±0.03		
	80	22.33b	7.50ab	2.82cd	0.62ef	4.54
	±0.33	±0.28	±0.17	±0.06		
	120	13.33f	5.00efg	1.59ghij	0.41g	3.87
	±0.33	±0.00	±0.29	±0.05		
	160	19.33c	6.00cde	2.87c	1.04a	2.75
	±0.66	±0.57	±0.17	±0.05		
	200	14.66e	5.33defg	1.25ij	0.66def	1.89
	±0.66	±0.44	±0.03	±0.07		
	400	4.33h	3.66hi	1.11j	0.46fg	2.41
	±0.33	±0.33	±0.01	±0.03		
	600	3.33h	3.00i	1.08j	0.40g	2.70
	±0.33	±0.00	±0.02	±0.01		
L.S.D	-	1.269	1.055	0.574	0.182	-

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$. F/D: fresh weight/dry weight. *Concentration as pollen grains equivalent

high concentrations of pollen extracts on shoot multiplication and rooting may due to high concentrations of plant growth substances in pollen extracts. The results were supported by many authors [14, 15, 5], who reported that pollens contain auxins and cytokinins. Finally, it could be concluded that pollen extracts can be used in the plant tissue culture medium as economic and natural source of plant growth substances instead of synthetic growth regulators.

SDS-PAGE of Explants of Tissue Culture: The proteins of explants resulted from mostly better treatments [BA (3 ppm), IBA+NAA (0.20+0.18 mg kg^{-1}), WPE (200 mg kg^{-1}), EPE (200 mg kg^{-1}) and MS] were extracted

Table 5: Quantitative analysis of explant protein subunits separated by SDS-PAGE

		Band						
Lane	Parameter	1	2	3	4	5	6	7
MS	MW	95.00	78.00	-	57.00	-	32.00	24.00
	Area%	12.43	14.95	-	36.37	-	18.70	17.52
IBA+ NAA	MW	95.00	78.00	65.00	57.00	50.00	32.00	24.00
	Area%	10.12	13.44	13.91	15.39	15.63	16.28	15.20
BA	MW	95.00	78.00	-	57.00	-	32.00	24.00
	Area%	13.03	15.55	-	35.25	-	18.92	17.22
WPE	MW	95.00	78.00	65.00	57.00	-	32.00	24.00
	Area%	10.62	14.50	15.55	27.01	-	17.13	15.17
EPE	MW	95.00	78.00	-	57.00	-	32.00	24.00
	Area%	12.95	16.14	-	38.50	-	16.74	15.64

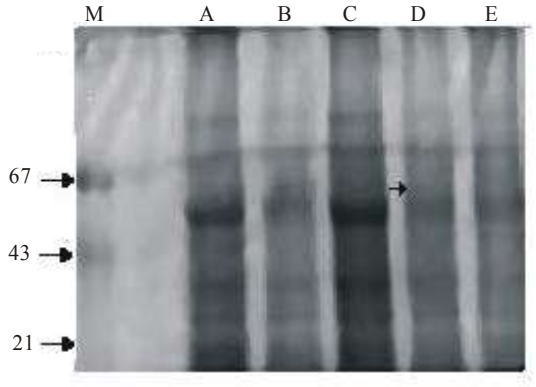

Fig. 1: SDS-PAG electrophoretic patterns of explant proteins obtained from tissue culture. Lane M: molecular weight marker (BSA; 67 kDa, egg albumin; 43 kDa and SBTI; 21 kDa), lane A: explants of MS medium, lane B: explants of IBA (0.20 ppm)+NAA (0.18 ppm), lane C: explants of BA (3 ppm), lane D: explants of water pollen extract (200 ppm) and lane E: explants of ethanol pollen extract (200 ppm)

and separated by SDS-PAGE. Figure 1 illustrates the SDS-PAG electrophoretic patterns of explant proteins. The data of MW and relative area percentage of each band are listed in Table 5. Five bands (subunits) with MWs of about 24, 32, 57, 78 and 95 kDa were detected in all explants. The obtained results revealed that there was additionally two bands with MW of about 50 and 65 kDa detected in explants of IBA+NAA treatment. Only additional band with MW of about 65 kDa was detected in explants of WPE treatment.

In general, the electrophoretic profile of subunits of explants obtained from different treatments was similar to the profile of MS explants. The additional subunits observed in IBA+NAA and WPE treatments may be due to the promoting effect(s) of auxins on rooting of explants. Together with the growth regulated standards, these tools can be used to visualize and compare complex mixture of proteins and to gain a large amount of information related to individual proteins involved in specific biological responses [16].

CONCLUSION

Finally, it could be concluded that palm pollen extracts can be used in the plant tissue culture medium as economic and natural source of plant growth substances instead of synthetic growth regulators.

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