0971-4693/94 US $ 5.00
Allelopathy Journal 23 (1): 51-62 (2009) *International Allelopathy Foundation* 2009
Tables: 3, Figs : 8

Effects of flavonols and phytohormones on germination and growth of petunia male gametophyte

L.V. KOVALEVA*, E.V. ZAKHAROVA, Y.V. MINKINA and A.S. VORONKOV

Timiryazev Institute of Plant Physiology
Russian Academy of Sciences, Botanicheskaya street 35, Moscow-127276, Russia
E. Mail: kovaleva_l@mail.ru

(Received in revised form: September 18, 2008)

ABSTRACT

We determined the contents of endogenous phytohormones and flavonols on *in vitro* germinating petunia (*Petunia hybrida* L.) male gametophyte and their effects on petunia pollen germination and pollen tube growth. The germination and growth of pollen tubes on the cultivation medium (containing 0.4 M sucrose and 1.6 mM H_3BO_3) increased the contents of IAA, ABA, gibberellins, cytokinins and flavonols (quercetin and kaempferol). The qualitative effects of exogenous compounds depended on their nature and concentration. All test compounds were most stimulatory at 10^{-12} M concentration. The applied exogenous ABA and gibberellin A_3 stimulated the germination and growth of male gametophyte at all test concentrations (10^{-12}–10^{-3} M). Gibberellin A_3, proved most stimulatory to growth of pollen tubes. IAA stimulated the germination and growth of male gametophyte at 10^{-12} – 10^{-8} M concentrations, whereas, the inhibition was observed at 10^{-4} – 10^{-3} M concentrations. The test flavonols, stimulated both processes at 10^{-12}–10^{-10} M concentrations and caused inhibition at 10^{-6}–10^{-3} M concentrations. Synthetic cytokinin 6-BAP at 10^{-12}–10^{-3} M concentrations inhibited both the germination and growth of pollen tubes. The 2,4-chlorphenoxy-2-methylpropionic acid (inhibitor of IAA transport) completely blocked both the processes, while the fluridone and paclobutrasol (known inhibitors of synthesis of ABA and gibberellins respectively), only inhibited the germination and growth of male gametophyte. In the presence of 2,4-chlorphenoxy-2-methylpropionic acid (inhibitor of IAA transport), exogenous ABA, gibberellin A_3 and flavonols did not stimulate pollen germination.

Key words: Flavonols, germination, growth, male gametophyte, phytohormones, *Petunia hybrida*

INTRODUCTION

In current biology, the selection of suitable cellular model systems to elucidate the mechanisms of allelopathic activity represents one of the major problems. The male gametophyte of plants (pollen) being unicellular organism may solve this problem, because (i) it germinates well on artificial nutrient mediums or in water (ii) releases many biologically active compounds including allelochemicals in the cultivation medium and (iii) is sensitive to many natural compounds (4, 7).

This study aimed to elucidate, whether petunia male gametophyte can be used as suitable model system for allelopathic studies. Hence, we (i). studied the effects of

*Correspondence author

flavonols (quercetin and kaempferol) and phytohormones (IAA, ABA, gibberellins and cytokinins) on pollen germination and pollen tube growth and (ii). determined the contents of endogenous test compounds in both mature pollen and germinating male gametophyte.

MATERIAL AND METHODS

This study consisted of two factors: **I. Plant materials**: 2. [(i).Intact petunia (*Petunia hybrida* L.) male gametophyte (pollen) and (ii). *In vitro* germinating petunia pollen] and **II. Cultivation mediums** 3 [(i). Water, (ii). Medium A: 0.4 M sucrose and 1.6 mM H_3BO_3 (pH 6.83) and (iii). Medium B: 0.4 M sucrose, 1.6 mM H_3BO_3, 1.3 mM $Ca(NO_3)_2$, 0.9 mM KNO_3, 0.8 mM $MgSO_4$ (pH 6.79) modified Brewbacker & Kwack medium (1)]. Plants were grown in soil in our greenhouse under natural illumination. All the experiments were done with freshly collected mature pollen.

In vitro* cultivation of petunia male gametophyte*: Two mg pollen was mixed with 2 ml of cultivation medium with glass rod, creating thin layer of pollen suspension in flasks (15 ml) hermetically closed with resin corks. The pollens were cultivated on different mediums for 6 h at 25-26^{o}C. The pollen grains germination (%) and length of pollen tubes were determined with light microscope equipped with ocular-micrometer up to 150-folds magnification. In each variant of experiments, the lengths of 100 germinated pollen tubes was measured.

Phytohormones*:* The contents of free forms of phytohormones [abscisic acid (ABA), indolyl acetic acid (IAA) and cytokinins] were determined with HPLC (8). Biological activity of gibberellins was determined in biotests using Frankland and Wareing method (3).

Free cytokinins*:* Free cytokinins were determined with ELISA using polyclonal antibodies against zeatin, zeatinriboside, isopentenyladenin and isopentenyladenosine after preliminary purification and HPLC fractioning of pollen extracts.

Flavonols and phenolic compounds*:* Freshly collected pollen (10 mg) was germinated for 6 h at 25-26^{o}C on the medium containing 0.4 M sucrose and 1.6 mM boric acid, washed with distilled water and fixed with 70% ethanol for 1 h under constant stirring. The ethanol extract obtained was filtered and tested for total content of phenolic compounds using Folin-Denis reagent after absorption of samples at 725 nm. Total content of flavonols was determined using 0.5% water solution of aluminium chloride and measuring absorption of samples at 415 nm. For both measurements, standard curves were obtained using rutin. In figures, the means obtained from 3-5 independent measurements and their standard deviations are presented. Difference between the means obtained for the experiment and the control was significant and assessed using Student's t - Test at $P < 0.05$.

Effects of exogenous flavonols and phytohormones on *in vitro* germination and growth of male gametophyte*:* In experiments testing action of exogenous phytohormones (IAA, gibberellin A_3 and ABA), synthetic cytokinin 6-BAP and flavonols (quercetin and kaempferol) on *in vitro* germination and growth of petunia male gametophyte, these

compounds were added at various concentrations (10^{-12}- 10^{-3} M) to the cultivation medium before the start of experiment, simultaneously with pollen sample. Figures represent the means and their standard deviations were obtained from independent experiments, (n = 6-10). Difference in significance was assessed using a Student's t - Test at $P < 0.05$.

RESULTS AND DISCUSSION

Composition of cultivation medium

A major factor influencing the germination and growth of male gametophyte is the composition of its cultivation medium. Upon selection of latter for petunia male gametophyte, we found in literature that essential factors for germination and growth of pollen tubes in angiosperms are carbohydrates and boron (component of plant cell wall) (2,9,11). After 6-h cultivation of male gametophyte on 0.4 M sucrose medium, pollen grains germination was 35% and pollen tube was 45 µm long, whereas, in cultivation medium of 1.6 mM H_3BO_3 these parameters were 30% and 120 µm, respectively (Fig. 1). When both sucrose and H_3BO_3 were mixed in the cultivation medium, pollen germination increased to 60% and pollen tube length achieved 200 µm (Fig. 1). Thus sucrose stimulated germination of pollen grains whereas boric acid stimulated both processes. Thus, an optimal composition of medium for petunia male gametophyte cultivation was found.

In water medium, pollen grains germination of both clones was 10-25% and pollen tubes length was 10-25 µm (Fig.2). In medium A, pollen germination was 62 and 48% for clones I and II, respectively, whereas, the maximal length of their pollen tubes was 200 and 250 µm, respectively (Fig.2). In medium B, pollen germination was 72 and 60% for clone I and II, respectively and maximal length of pollen tubes was 270 and 370 µm for clones I and II, respectively (Fig.2).

The use of three cultivation mediums allowed us to investigate the effects of test phytohormones on germination and growth of petunia pollen tubes. The test phytohormones were most efficient on the medium A, hence, major part of our study was done in this medium.

Phenolic compounds, flavonols and phytohormones

In medium A, the *in-vitro* germination of petunia male gametophyte cultivated for 4 h, the soluble phenolic compounds contents were variable (40-50 mg/g. FW) (Fig. 3). In the first 2 h after cultivation, flavonols increased (15-30%) and thereafter, decreased gradually till 5 h. Likewise, phytohormones (IAA, gibberellins and cytokinins) contents also increased (Table 1).

During the *in vitro* pollen tube growth, we observed the release of hormones into the cultivation medium, that suggests their synthesis in growing male gametophyte.

Effects of exogenous flavonols and phytohormones

Application of phytohormones to the cultivation medium A significantly influenced the germination of petunia male gametophyte (Fig. 4). However, flavonols were less effective. Gibberellin A_3 and ABA at 10^{-12} M to 10^{-3} M concentrations markedly

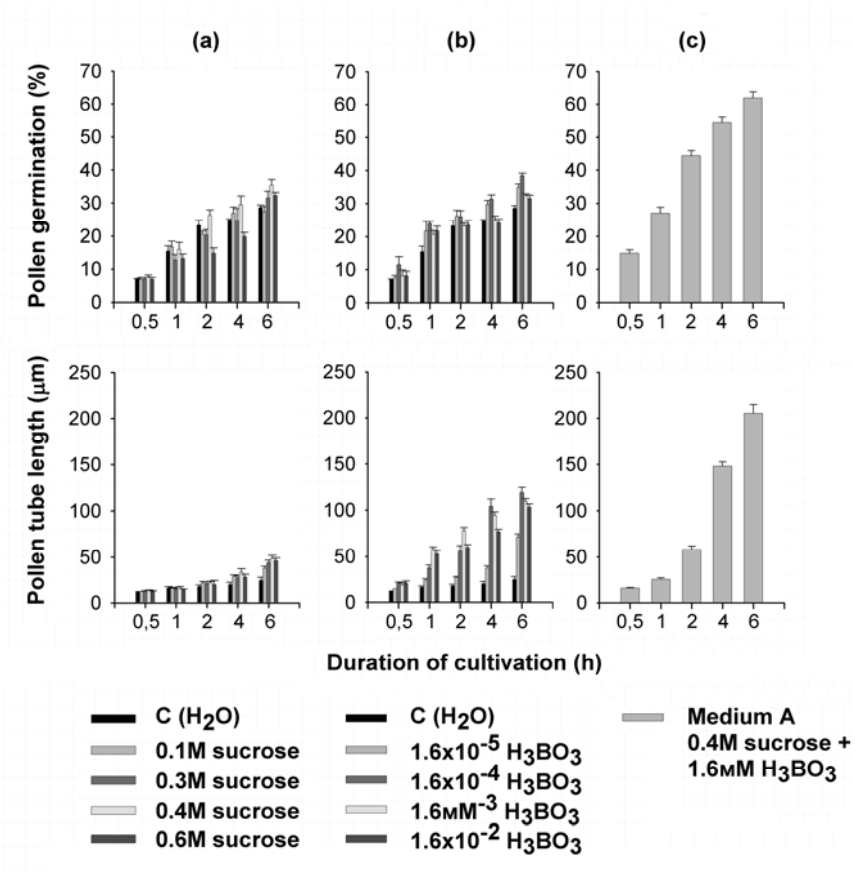

Figure 1. Petunia pollen germination and pollen tube growth on medium containing (a) sucrose, (b)H_3BO_3, (c) 0.4 M sucrose + 1.6 mM H_3BO_3

Table 1. Dynamics of phytohormones contents in germinating petunia male gametophyte (medium A)

Phytohormones, ng/g FW	Hours after cultivation (h)				
	0	2	4	6	8
ABA	1000±58.6	0	0	0	0
Gibberellins	48 ± 3.5	98 ±12.7	147 ± 14.5	196 ± 21.6	124 ± 12.8
IAA	35 ± 2.1	44 ± 3.7	10 ± 0.5	10 ± 0.5	12 ± 2.2
Cytokinins	28 ± 2.6	51 ± 6.8	30 ± 2.7	30 ± 2.7	43 ± 2.7

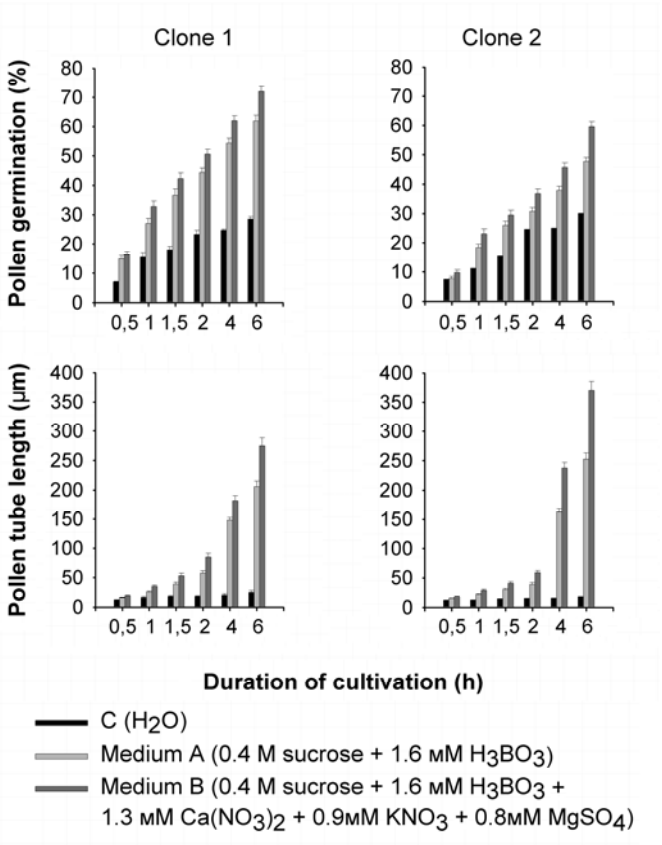

Figure 2. Pollen germination and pollen tube growth in two petunia clones on the three mediums of cultivation

stimulated the germination of pollen grains, whereas, IAA at 10^{-12} M to 10^{-10} M concentrations were stimulatory but higher concentrations (10^{-4} – 10^{-3} M) were inhibitory. All test compounds were stimulatory at 10^{-12} M concentration to germination of male gametophyte on the medium A. While synthetic cytokinin 6-BAP at 10^{-12} M to 10^{-3} M concentrations, inhibited the germination. The flavonol (kaempferol) and phytohormones (IAA, gibberellin A_3 and cytokinin 6-BAP) at 10^{-12} M concentration influenced the germination and growth of petunia pollen tubes on three cultivation mediums (Figs. 5-8).

On the water, kaempferol and phytohormones insignificantly stimulated the pollen germination and pollen tube growth (Fig. 5 and Fig. 6). On medium A, kaempferol stimulated the pollen germination by 60% and pollen tube growth by 30% at 4-6 h after the cultivation (Fig. 5). On medium B, kaempferol insignificantly stimulated the pollen germination but inhibited the pollen tube growth (Fig. 5).

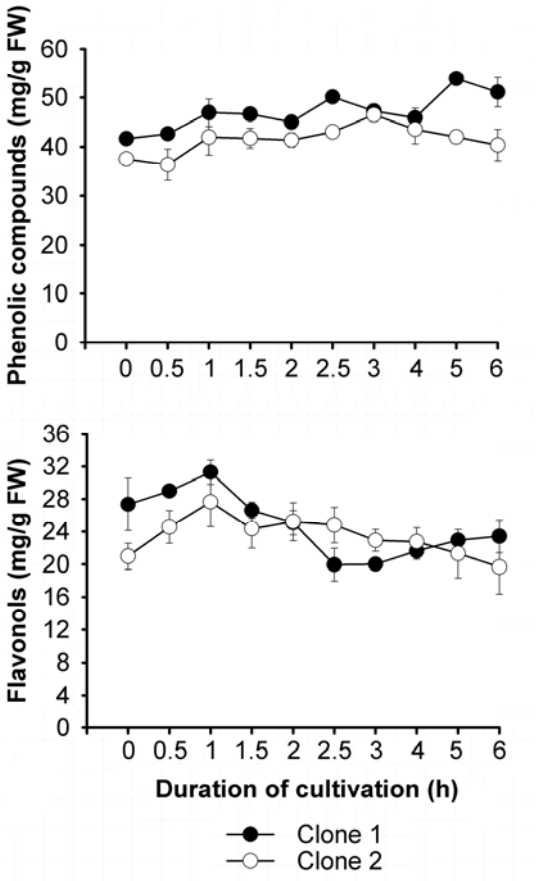

Figure 3. Content of phenolic compounds and flavonols in germinating petunia pollen

On medium A, the gibberellin A_3, ABA and IAA stimulated these processes, but the latter two hormones greatly influenced the pollen grains germination than pollen tube growth (Fig. 7).

Contrarily, on the medium B [0.4 M sucrose, 1.6 mM H_3BO_3, 1.3 mM $Ca(NO_3)_2$, 0.9 mM KNO_3, 0.8 mM $MgSO_4$], the phytohormones slightly stimulated the pollen germination, but inhibited the pollen tube growth, except that gibberellins also slightly stimulated the pollen tube growth (Fig. 8).

Gibberellin A_3: Gibberellin A_3 at 10^{-12} – 10^{-3} M concentrations stimulated both pollen germination and pollen tube growth. In first 2.0 h after cultivation, the pollen germination was stimulated 2-3 folds (Table 2), while 2-3-folds stimulation in pollen tube growth

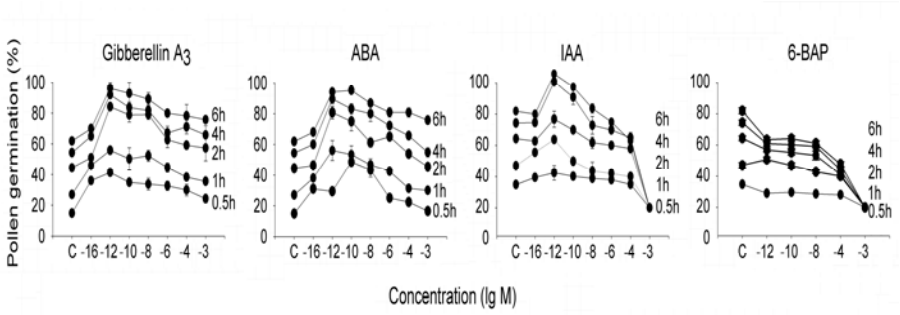

Figure 4. Effects of phytohormones on petunia pollen germination.

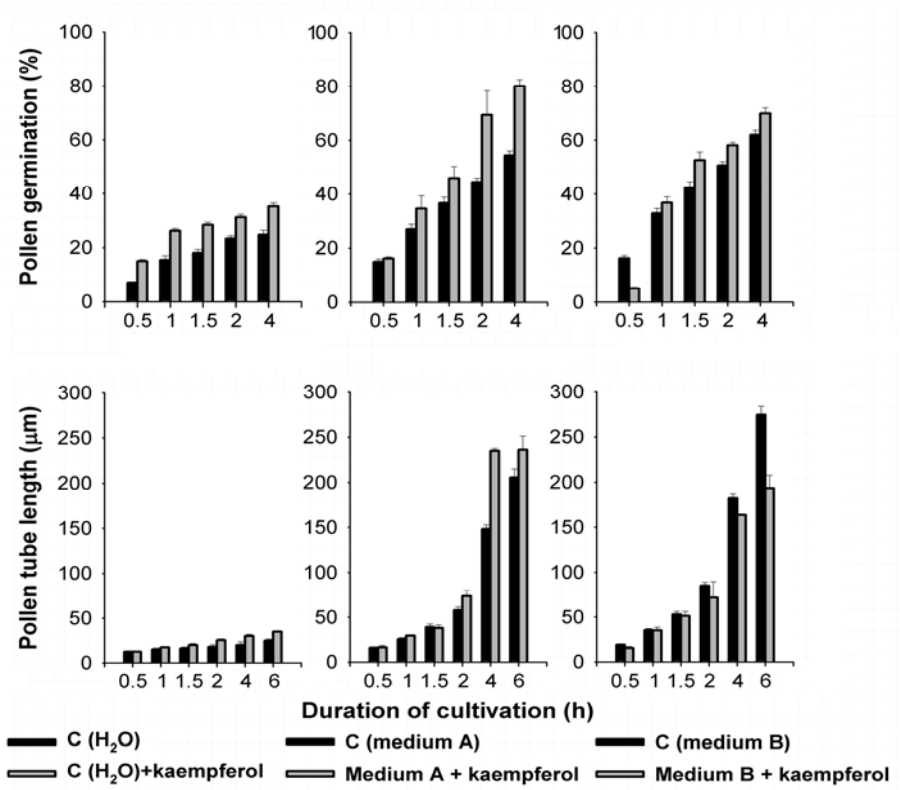

Figure 5. Effects of kaempferol on pollen germination and pollen tube growth on three mediums of cultivation

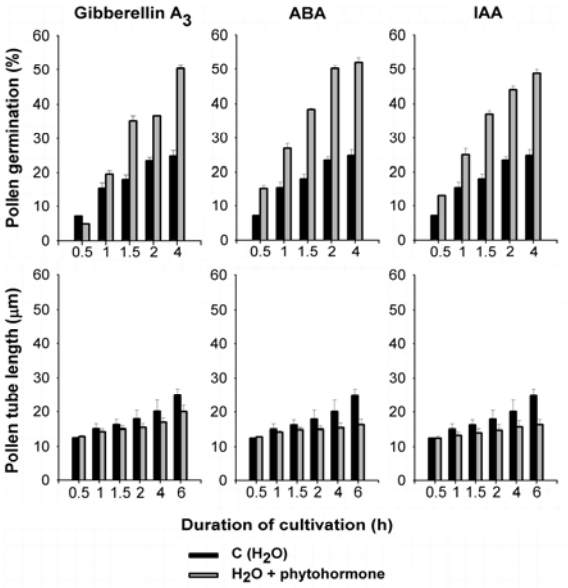

Figure 6. Pollen germination and pollen tube growth on the water with phytohormones.

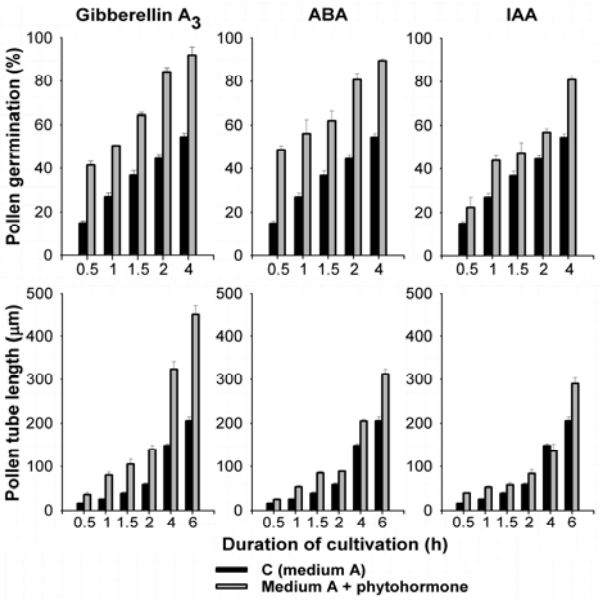

Figure 7. Pollen germination and pollen tube growth on the medium A with phytohormones

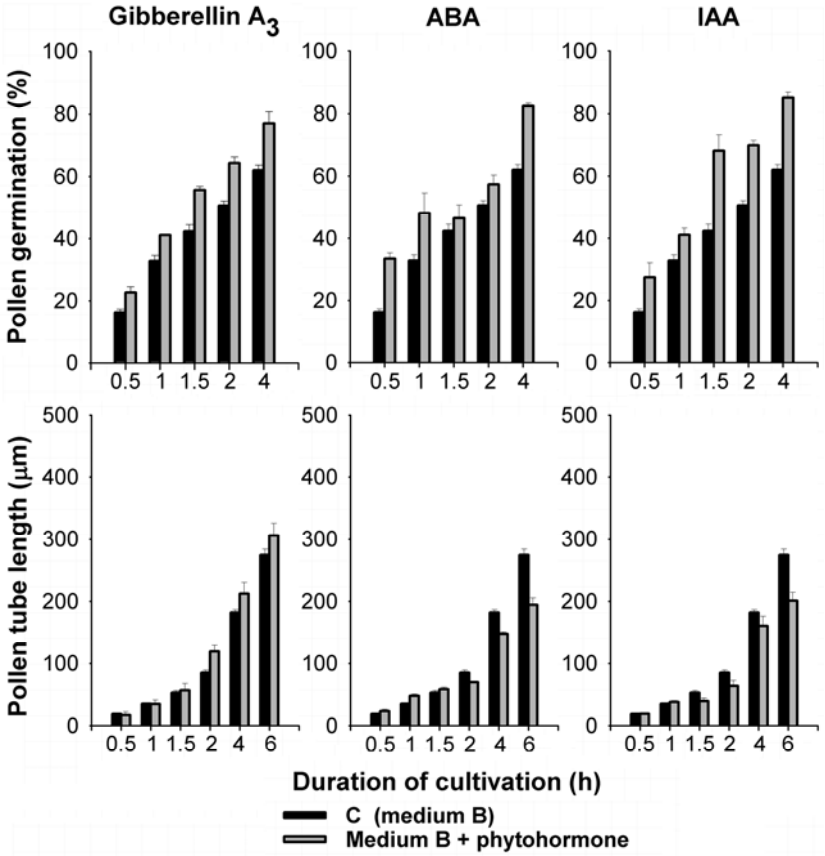

Figure 8. Pollen germination and pollen tube growth on the medium B with phytohormones.

Table 2. Effects of exogenous phytohormones on *in vitro* germination of petunia pollen grains 0.5 h after their cultivation on medium A (an intensity of this process on the medium A = 100% (C))

Phytohormone	10^{-12} M	10^{-10} M	10^{-8} M	10^{-6} M	10^{-4} M	10^{-3} M
Gibberellin A_3	278	235	227	219	200	163
ABA	327	291	198	168	151	112
IAA	151	136	127	121	101	0
6-BAP	100	83	75	70	65	0

occurred after 6 h (Table 3). After 6-h cultivation, the pollen grains germination was 90% and pollen tubes length was 450 μm. However, the pollen did not germinate after addition of paclobutrazol (a known inhibitor of gibberellin synthesis), to the cultivation medium.

Table 3. Effects of exogenous phytohormones on *in vitro* growth of petunia pollen tube growth (μm) 0.5 h after pollen cultivation on medium A (an intensity of this process on the medium A=100% (C))

Phytohormone	10^{-12} M	10^{-10} M	10^{-8} M	10^{-6} M	10^{-4} M	10^{-3} M
Gibberellin A_3	299	228	228	197	157	118
ABA	259	165	157	118	118	94
IAA	252	252	238	239	220	0
6-BAP	100	100	100	100	100	0

ABA: ABA at 10^{-12} M concentration significantly stimulated the pollen germination (up to 90%) (Table 2), but was less stimulatory to pollen tube growth (upto 300 μm) (Table 3). The ABA at 10^{-12}M concentration caused maximum stimulation (3-folds) in pollen germination in first 30 min and 2-3 folds stimulation in pollen germination and pollen tube growth 1.0 h after cultivation. The pollen germination was inhibited after addition of fluridone (inhibitor of ABA synthesis) to the cultivation medium.

IAA: IAA was less stimulatory to pollen germination (up to 80%) than gibberellin A_3 (Table 2) and stimulated the pollen tubes length to 300 μm (Table 3). IAA at 10^{-12} M concentration stimulated the pollen germination and pollen tube growth by 1.5 and 2-2.5 times, respectively. The 2,4-chlorphenoxy-2-methylpropionic acid (known inhibitor of IAA transport), at 10^{-3} M concentration completely blocked the pollen germination in both absence or presence of phytohormones (ABA and gibberellin A_3) in the cultivation medium. The combined application of IAA and quercetin to the cultivation medium, increased the pollen germination than addition of only one compound.

Thus, the test phytohormones (ABA, gibberellin A_3 and IAA), stimulated the petunia pollen grain germination and pollen tube growth, whereas, synthetic cytokinin 6-BAP inhibited these processes. Gibberellin A_3 and ABA were most stimulatory to pollen germination at 10^{-12} M concentration.

These results about the effects of exogenous flavonols on germination and growth of petunia pollen tubes agree with literature (6,10,11). These findings showed that flavonols act like phytohormones as plant growth regulators during male gametophyte development. Based on present and recent researches (5), we put forward the hypothesis that polar transport of IAA influences the germination and growth of male gametophyte, while ABA regulates their intracellular osmotic pressure during these processes. Our experiments also showed the involvement of gibberellins in regulation of pollen tube growth.

REFERENCES

1. Brewbaker, J.L. and Kwack, B.H. (1964). The calcium ion and substances influencing pollen growth. In: *Pollen Physiology and Fertilization.* (Ed., H.F. Linskens). Elsevier Amsterdam. pp. 145-151.
2. Holdaway-Clarke, T.L., Weddle, N.M., Kim, S., Robi, A., Parris, C., Kunkel, J.C. and Hepler, P.K. (2003). Effects of extracellular calcium, pH and borate on growth oscillations in *Lilium formosanum* pollen tubes. *Journal of Experimental Botany* **54:** 65-72.
3. Frankland B. and Wareing P. (1960). Effects of gibberellic acid on hypocotyls growth of lettuce seedlings. *Nature* **185**: 4700-4708.

4. Gaur, S., Rana, A. and Chauhan, S.V.S. (2007). Pollen allelopathy: Past achievements and future approach. *Allelopathy Journal* **20**:115-126.
5. Kovaleva, L.V. and Zakharova, E.V. (2003). Hormonal status of pollen-pistil system at the progamic phase of fertilization after compatible and incompatible pollination. *Sexual Plant Reproduction* **16**:191-196.
6. Mo, Y., Nagel, C. and Taylor, L.P. (1992). Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proceedings National Academy of Science, USA* **89**: 7213-7217.
7. Roshchina, V.V. (2004). Cellular models to study the allelopathic mechanisms. *Allelopathy Journal* **13**: 3-16.
8. Skorobogatova, I.V., Zakharova, E.V., Karsunkina, N.P., Kurapov, P.B., Sorkina, G.L. and Kislin, E.N. (1999). Changes in the hormone content in barley seedlings during their development. *Agrokhimiya* **8**: 49-53.
9. Steer, M.W. and Steer, J.M. (1989). Pollen tube tip growth. *New Phytologist* **111**:323-358.
10. Vogt, T., Wollenweber, E. and Taylor, L.P. (1995). The structural requirements of flavonol that induce pollen germination of conditionally male fertile Petunia. *Phytochemistry* **38:** 589-592.
11. Ylstra, B., Touraev, A., Moreno, R.M.B., Stoger, E., van Tunen, A.J., Vicente, O., Moll, J.N.M. and Heberle-Bors, E. (1992). Flavonols stimulate development, germination and tube growth of tobacco pollen. *Plant Physiology* **100:** 902-907.