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Short Communication

Inhibition of Gamete Formation by Cycloheximide in Pollen Tubes of *Impatiens balsamina*

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Summary. Cycloheximide (CH) inhibited the division of generative cell in pollen cultures of *Impatiens balsamina* in concentrations of 1–200 μg/ml without affecting pollen germination or pollen tube growth. This inhibition was reversible up to 3 h in hanging drop cultures and up to 6 h in test tube cultures by replacement of the medium with a CH-free one.

Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, does not affect pollen germination and the early growth of the pollen tube in many species but inhibitors of protein synthesis such as cycloheximide, puromycin and chloramphenicol greatly reduce both pollen germination and pollen-tube growth in *Tradescantia* and *Lobelia* (Dexheimer, 1966, 1968; Mascarenhas, 1971). As far as we know these are the only studies of effects of protein-synthesis inhibitors on these processes. This communication describes the effects of cycloheximide (CH) treatment on pollen germination, pollen-tube growth, and division of the generative cell in *Impatiens balsamina*.

Plants of *Impatiens balsamina* L. were grown under field conditions obtaining in Delhi from August to October. Pollen grains were always collected from freshly dehisced anthers. At the time of shedding, the pollen is two-celled (Fig. 1A) and the generative nucleus is usually in the pro-metaphase stage of mitosis.

The pollen grains were germinated in hanging drops or in liquid culture in test tubes. The culture medium consisted of 10% sucrose, 100 mg/l boric acid, 300 mg/l calcium chloride, 200 mg/l magnesium sulphate and 100 mg/l potassium nitrate (Brewbaker and Kwack, 1963). Cycloheximide was added at 1, 10, 50, 100 and 200 μg/ml. Hanging-drop cultures (16 per treatment) were raised in cavity slides in four replicates. At least 200 pollen grains and 50 pollen tubes were scored in each replicate for germination and tube length, respectively, and the data pooled for each treatment. The cultures were maintained under laboratory conditions.

Test-tube cultures were raised in 7.5 × 1.5 cm test tubes with 1 ml of medium. Anthers from 2 flowers were shaken in the medium to release the pollen grains, and the anthers removed. The culture tubes were put on a horizontal shaker (100 rpm) and maintained at room temperature. Three cultures were raised for each treatment and the experiment was replicated thrice.

When CH medium was to be replaced with CH-free medium, the procedures used were as follows. With hanging-drop cultures, the cover slip with the hanging

drop was removed and placed in an inverted position. The medium was carefully removed either with a micropipette or with a piece of filter paper, leaving behind the pollen tubes. The latter were thoroughly washed 4–6 times with fresh CH-free medium, and the coverslip with a drop of this medium was repositioned on the cavity slide. In the case of test-tube cultures the CH medium was replaced by CH free medium by means of centrifugation (1500–2000 rpm, 2 min). In either type of culture, fresh medium containing CH was used for both washing and reincubation as control.

For studying gamete formation, a suspension of pollen tubes was placed on a slide and stained with 1% propionocarmine. At least 120 tubes were scored for gamete formation from each treatment.

Pollen germination occurred 10–15 min after culture and was consistently good in all treatments. Table 1 gives the percentage of pollen germination and length of pollen tubes in hanging drop cultures. Even at the highest concentration tried (200 μg/ml), CH failed to affect either germination or growth of the pollen tube. Except at 200 μg/ml CH, much of the pollen tube growth had occurred in the 1st hour, very little during the 2nd hour, and nil beyond 2 h. At 200 μg/ml there was considerable growth of the pollen tubes during the 2nd hour also. In the control medium, the vegetative nucleus and the generative cell had migrated into the pollen tube after 30 min of culture. During the next 30 min, 62% of the pollen tubes had formed gametes, and almost all pollen tubes had gametes 2 h after culture. Fig. 1 B illustrates the condition after 3 h culture of the pollen. In contrast, in all CH treatments (including 1 μg/ml) no gametes were observed even after 9 h culture. The division of the generative cell was arrested at metaphase and 7 dot-like chromosomes could be observed (Fig. 1 C). In well-stained pollen tubes, the vegetative nucleus was also faintly recognizable, in both control and CH treatments.

To find out whether or not the effect of CH in arresting the division of the generative cell was reversible, the CH medium was replaced with CH-free medium. The results are summarized in Fig. 2 A. When the CH treatment was confined to 3 h, recovery of the generative cell

Table 1. Effect of CH on pollen germination and pollen tube growth in hanging-drop cultures

CH conc. (μg/ml)	Pollen germination (%)	Pollen tube length (μm)	
	1 h	1 h	2 h
0 (control)	97.3	603.7	760.8
1	98.2	625.5	766.5
10	98.1	678.0	782.4
50	97.5	639.0	726.5
100	97.6	619.5	766.2
200	97.7	543.0	787.5

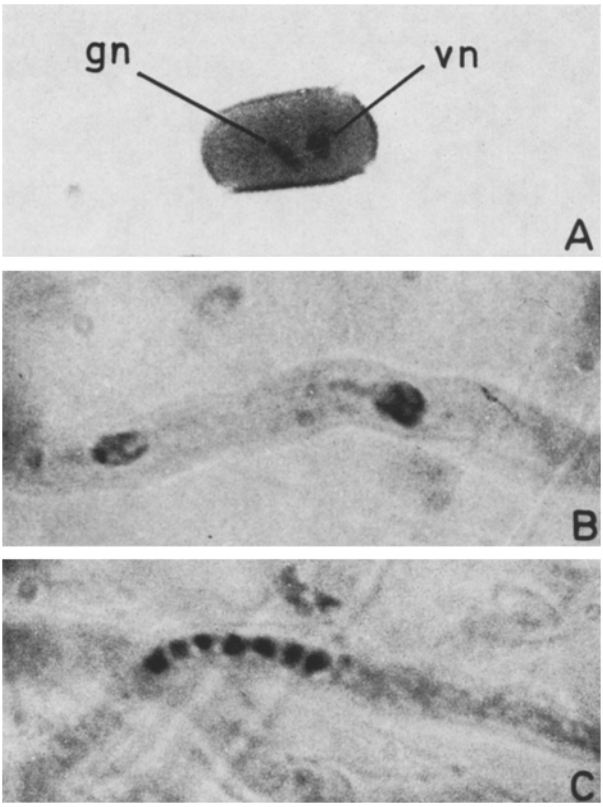

Fig. 1 A—C. Propionocarmine squash preparations of pollen grain and pollen tubes. A Pollen grain at culture showing the generative nucleus (*gn*) and the vegetative nucleus (*vn*). × 600. B Part of pollen tube 3 h after culture on CH free medium showing the gametes. × 1666. C Part of pollen tube 3 h after culture on CH medium. Division of the generative cell has been arrested at metaphase. × 1916

division was almost complete. Even at 200 μg/ml of CH, 77.5% of the tubes showed gamete formation. In the control (*i.e.*, washing and re-incubation in CH medium), however, there was no gamete formation irrespective of the concentration of CH used. When the CH treatment was extended to 6 h, there was no recovery of the generative cell division, except that in 1 μg/ml 12.5% of the tubes showed gamete formation.

Working with *Tradescantia paludosa*, Mascarenhas (1966) found that the inhibitory effect of actinomycin D could not be recognized in drop cultures, presumably since the growth of the pollen tubes in such cultures was limited owing to paucity of the medium. When the pollen tubes were grown in shake cultures which allowed greater tube growth, the inhibitory effect of actinomycin D was apparent.

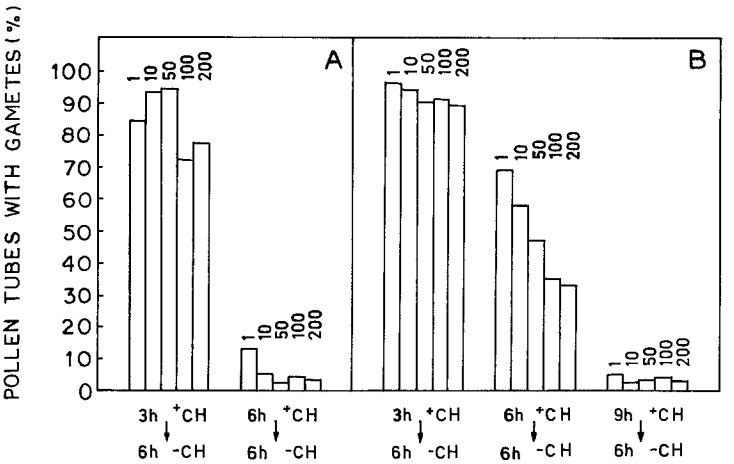

Fig. 2 A and B. Recovery of the generative cell division after different periods of CH treatment in hanging-drop (A) and test-tube cultures (B). Numbers above bars = concentration of CH in μg/ml. As not more than 2% of the tubes showed gamete formation in the controls for different treatments (in which fresh CH medium was used for washing and reincubation) these data are not presented

To find out whether a similar situation prevailed in our study, the extent of pollen tube growth in test-tube cultures was examined. In general the length of the pollen tubes was greater in test tube cultures than in the hanging-drop cultures. Although no accurate measurements of the lengths of pollen tube in the treatments was possible because of intertwining, no significant differences were noted. In all treatments the tube length exceeded 1500 μm. Thus, CH does not seem to inhibit the growth of the pollen tubes even in test-tube cultures.

The behaviour of the generative cell in test-tube cultures was also comparable to that in hanging-drop cultures. Gamete formation occurred only in cultures that were raised on CH-free medium; in all other treatments the division of the generative cell was arrested at metaphase. The only difference was that recovery of the generative cell division occurred if the CH medium was replaced not only after 3 h, but also after 6 h of culture. although to a lesser degree (Fig. 2 B). Extending the CH treatment to 9 h resulted in irreversible inhibition of the generative cell division. In the controls (reincubation in fresh CH medium), the percentage of pollen tubes showing gamete formation was negligible.

In *Tradescantia paludosa*, both CH and puromycin greatly reduced pollen germination and pollen-tube growth (Mascarenhas, 1971). Chloramphenicol was reported to be inhibitory to both these processes in *Lobelia* (Dexheimer, 1966). Based on these results Mascarenhas (1971) suggested that proteins synthesized in the initial stages of pollen germination were required for pollen germination and initial tube growth. Our results with *Impatiens balsamina* differ from these earlier studies. CH affected neither pollen germination nor pollen-tube growth in concentrations of 1 to 200 μg/ml. Thus the activities of the vegetative cell

were not affected by CH. CH, however, specifically arrested the division of the generative cell at all concentrations tested. This inhibition was reversible for 3 h in hanging-drop cultures, and for 6 h in test-tube cultures. This difference can be probably explained on the basis of continued growth of pollen tubes in test-tube cultures and their greater metabolic activity.

It is difficult to explain the differential response of vegetative and generative cells of *Impatiens* pollen grains to CH. Recently Arora *et al.* (1970) have observed a pronounced effect of CH in arresting the metaphase in root tips of *Vicia faba*. Many effects of CH are due to inhibitory effect on protein synthesis at the ribosomal level. However, Ellis and MacDonald (1970) showed that CH can affect cellular metabolism also independent of protein synthesis, and CH may not be a specific protein synthesis inhibitor in higher-plant tissues in general (MacDonald and Ellis, 1969). Boulter (1970) suggested that CH-mediated inhibition of protein synthesis may be species specific. No information is available on the synthesis of proteins during pollen germination and subsequent pollen-tube growth in *Impatiens*. To understand the mechanism of the action of CH on the division of the generative cell in *Impatiens* it is essential to determine whether protein synthesis occurs during those processes and whether it is sensitive to CH.

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