Acta Biol. Szeged. 31, pp. 49—53 (1985)

RAPID DETERMINATION OF POLLEN FERTILITY OF TWO INSECT POLLINATED PLANT SPECIES BY STAINING WITH THE AID OF PROLINE—ISATIN REACTION

G. PÁLFI and S. GULYÁS

(Received: March 20, 1984)

Abstract

A new rapid staining method has been elaborated for the pollens of *Chrysanthemum leucanthemum* and *Robinia pseudacacia* which can be used to indicate their fertility. With this staining fertility grade of the pollen grains can be seen by different colours. The staining can be performed on living and on fixed pollens as well. Results of the new method is supported by the in vitro observed germination per cent of the pollens and the proline content of their extracts. In marguerite the greatest number of fertile pollens can be collected when the flowers of the outermost circle of the capitulum are open, and fertility of the pollens of the flowers of the inner circles opening day by day centripetally diminishes gradually. The new method can be applied only when the pollen extracts contain at least 1% proline, in the dry matter. Proline content of the pollen extracts of *Helianthus annuus* remains below 0.03%, therefore fertility of the pollens of this species can not determined with the aid of our staining method.

Key words: Chrysanthemum, Helianthus, Insect-pollination, Isatin-reaction, Pollen grains, *Robinia.*

Introduction

Proline content of fertile pollens is extremely high, higher than the amount of the other free amino acids alltogether. Quantity of proline positively correlated with germination per cent and fertility of the pollens (TUPY, 1964; LINSKENS and SCHRAUWEN, 1969; HESLOP-HARRISON, 1971; AHOKAS, 1978; ZHANG et al., 1982).

These results were also proved by RAI and STOSKOPF (1977) on wheat and YAMADA and KONO (1977) on rice and recently by us on corn and rye (PÁLFI et al., 1981; PÁLFI and PÁLFI, 1982; PÁLFI and KÖVES, 1984).

According to HESLOP—HARRISON (1971, 1979), LINSKENS (1974), STANLEY and LINSKENS (1974), MASCARENHAS (1975), ZHANG et al. (1982) and ZHANG and CROES (1983) the important role of proline during germination is its activating effect on respiration and citrat cycle, it is a significant nitrogen source, it regulates water economy, keeps the enzymes in their active form and it is an important component of the proteins of pollen and germ-tube wall in the form of hydroxyproline. The role of proline in the elongation of germ-tube was examined by other authors as well (DASHEK and HARWOOD, 1974; BRITIKOV, 1975; DASHEK and MILLS, 1981).

Pollens of *Lilium longiflorum* were germinated at extremely high and extremly low temperatures in a medium containing ^{14}C-labelled proline (ZHANG and CROES, 1983). The pollens taking up exogeneous proline germinated considerably better than those which were put in the medium without proline. It was concluded that high proline content of the pollens gave them resistance in the case of unfavourable

temperatures and on this way increased the chances of fertilization. Similar results were obtained in the case of drought stress, low temperature treatments and high salt content of the medium by PÁLFI and JUHÁSZ (1969).

Based on the work of LINSKENS (1974), STANLEY and LINSKENS (1974), HESLOP—HARRISON (1971, 1979), DASHEK and MILLS (1981) and ZHANG et al. (1982) we elaborated a rapid staining method using an isatin reagent of new composition, which indicates the proline content of the grains and through this their grade of fertility with conspicuous colours. The results of the new staining procedure were compared with the proline content and in vitro germination per cent of pollens collected at the same time. The outer and inner structure and physiology of dinuclear pollens (dinucleotides) are entirely different from those of the trinucleotide-type pollens which are mostly insect-carried (HOEKSTRA and BRUINSMA, 1979).

Now we pretent our results obtained by isatin staining if trinuclear pollens of three insect-pollinated plant species.

Material and methods

Pollens of the composites marguerite *(Chrysanthemum leucanthemum* L.*)* and sunflower (*Helianthus annuus* L. cultivar Kisvárdai) and the leguminous robinia *(Robinia pseudacacia* L.*)* were investigated. In the case of the marguerite first pollens of the flowers of the outermost circle where flowering begins were collected and thereafter dayly that of the flowers of the inner circles as flowering advanced centripetally. In the case of *Robinia* racemes were collected and pollens were separated in the laboratory. In vitro germination of the pollens of three species was also investigated, Composition of the agar-media used is already publiched elsewhere (PÁLFI and KÖVES, 1984).

Proline content of the pollen extracts was determined according to BATES et al. (1973) and the results were controlled with the method of ASPINALL et al. (1973).

A new composition of the isatin-reagent was worked out for pollen staining: 1 ml glacial acetic acid and 1 ml glycerol was added to 100 ml acetone and 0.50 g isatin was dissolved in this mixture (glycerol is not always necessary).

Staining of the pollen grains was microscopically evaluated, therefore the procedure was performed on slides. 2—20 mg pollen was placed on the middle of a slide with the aid of a lancet, two drops of the reagent was added and mixed with the grains till acetone evaporates. This procedure was repeated two times with one drop of reagent. Thereafter the slide was put in *a.* 90 °C exsiccator for ten minutes to develop colours. After cooling down superfluous stain around the pollens was cleaned off with wet cotton wool. A drop of paraffin oil was added to the adhered pollens, they were disperged in it with the aid of a glass rod and the dispersion was spread with a lancet in the middle of the slide. Finally it was covered with a cover glass and slightly pressed.

In the case of living pollens they are fixed by the acetone treatment and the high temperature of colour development. When staining is not performed on the day of collection, pollens should be fixed at 90 °C. The entirely desiccated pollens are stored in hermetically sealed vessels in the dark and their staining can be performed after 12—16 weeks well.

Results and discussion

It can be seen on the microphotos of Figure 1. that pollens of the outermost circle of the capitulum of marguerite are nearly all stained black. These pollens contain the highest quantity of proline and therefore these are for the most part fertile. Evaluating the staining degree of the pollen grains on five fields of sight the per cent of fertility also can be experimentally determined. Advancing to the centre of the capitulum in zones of flowering the proportion of black or dark blue stained pollen grains continually decreases. It means that the fertility grade of pollens lessens in

this direction. Poorly fertile pollen grains stain light blue or light green, sterile pollen grains remain yellow or stain brown (on the black-and white photos different shades of grey). The greatest number of sterile pollen comes from the centre of the capitulum.

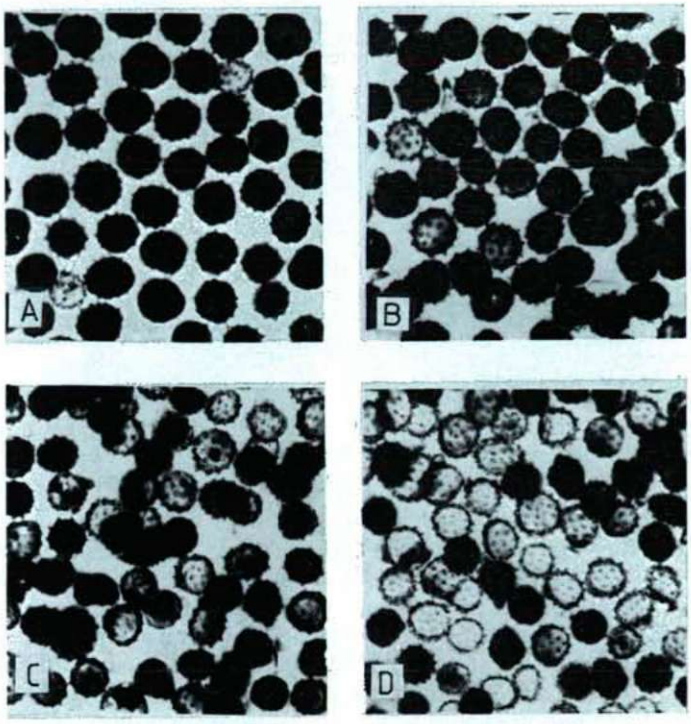

Fig. 1. Approximate determination of fertility of marguerite pollens by rapid staining with the aid of the proline-isatin reaction. Fertile pollens are black, dark grey shades show poor fertility, the light-coloured pollens are sterile. Magnified 300×. The fertility per cent is A)=98%; B)=81%; C)=54%, and D)=42%.

The results of in vitro germination and fertility experiments in the case of the pollens of three species obtained with three different methods are compared on Table 1.

It can be seen on the table that the pollens of *Robinia pseudacacia* germinate rather well in vitro and also their proline content is high. The per cent of grains stained dark (on the Figure they are black) is higher than the per cent of germination.

Pollens of *Helianthus annuus* germinate in higher per cent than those of the former species. The good germination shows that the collection of pollens in living state was successful. Proline content of the extract of sunflower pollens is less than 0.03%. Due to this low proline quantity the cell walls of the grains could not be stained with isatin.

Fertility grade of the *Chrysanthemum leucanthemum* pollens shows identical

Table 1. Comparison of the in vitro germination of pollens, the proline content of the pollen extracts, and the data of staining of the pollen grains with isatin in the case of three insect-pollinated species. Proline content = proline concentration of the pollen extracts in per cent of the dry matter. Staining reaction with isatin is positive (and the pollen is fertile) when the pollen grain becomes dark blue or black. The values in the Table are means of 3 repetition; deviation of the repetitions from the mean is less than ±5%.

Species	Germination per cent in vitro	Proline content %	Positive staining with isatin; %
Robina; *Robinia pseudacacia*	68	1.74	78
Sunflower *Helianthus annuus*	76	0.026	—
Marguerite; flowers of the outermost circle of the capitulum	85	2.15	98
Marguerite; flowers of the second circle of the capitulum	77	1.79	81
Marguerite; flowers of the third circle of the capitulum	47	1.38	54
Marguerite; flowers of the middle of the capitulum	39	1.06	42

tendencies in the three different investigations. The highest values were obtained in pollens collected in the outermost circle of flowers of the capitulum. Proceeding centripetally in the capitulum fertility gradually diminishes and the greatest quantity of sterile pollens was found in the centre. Fertility grades of the pollen grains nearly correspond to the proline levels of the pollen extracts and to the germination per cent in vitro; the staining reaction gives always a higher value than the germination per cent. This can be so interpreted that the staining with isatin indicates the possibility which could be realized if the pollen grains had been living. The investigation of the in vitro germination should be performed on fresh pollens and this can not be perfectly realized in every case.

Our results show that not all insect-pollinated plants have pollens with high free proline content. E.g. *Helianthus annuus* pollens contain very small amount of proline, *Helianthus* is not a proline-type pollen.

Pollens of proline-type species contain more than 1.0% proline in the dry weight. This conclusion was obtained with the pollens of 16 inbred lines and 5 hybrids of corn (PÁLFI et al., 1981; PÁLFI and PÁLFI, 1982), of 5 cultivars of rye (PÁLFI and KÖVES, 1984) and with the pollens of 3 insect-pollinated species in this paper; proline content of the extracts of these pollens was determined and the pollens were investigated with the isatin reagent as well. Other papers also support our results (TUPÝ, 1964; LINSKENS, 1974; STANLEY and LINSKENS, 1974; BRITIKOV, 1975; HESLOP—HARRISON, 1971, 1979; DASHEK and MILLS, 1981; ZHANG et al., 1982; etc.).

It can established that fertility of the pollens of different plant species as well as of different cultivars of these species can be approximately determined on the basis of their proline content with the aid of the rapid isatin staining — if the pollen of species is of the "proline-type". Success of the determination is not influenced by the mode of pollination (by wind or by insects).

References

AHOKAS, H. (1978): Cytoplasmic male sterilit of barley. II. Physiology and anther cytology of msml. — Hereditas. Lund. *89,* 1—21.

ASPINALL, D., SINGH, T. N. and PALEG, L. G. (1973): Stress metabolism. — Austral, J. Biol. Sci. *26,* 319—327.

BATES, L. S., WALDREN, R. P. and TEARE, I. D. (1973): Rapid determination of free proline for water-stress. — Planta and Soil. *39,* 205—207.

BRITIKOV, E. A. (1975): Biologicheskaya roly proline. — Izd. Nauka. Moskva.

DASHEK, W. V. and HARWOOD, H. I. (1974): Proline, hydroxyproline and lyli pollen tube elongation. — Ann. Bot. *38,* 947—959.

DASHEK, W. V. and MILLS, R. R. (1981): Proline metabolism by germinating *Lilium longiflorum.* — Acta Soc. Bot. Pol. *50,* 51—66.

HESLOP-HARRISON, J. (1971): The pollen wall. Structure and development. In: Pollen: Development and Physiology, (Ed. HESLOP—HARRISON, J.) pp. 75—98, London: Butterworth.

HESLOP-HARRISON, J. (1979): Aspects of the structure, cytochemistry and germination of the pollen of rye *(Secale cereale).* Anna. Bot. London *44,* Suppl. 1. 1—47.

HOEKSTRA, F. A. and BRUINSMA, J. (1979): Protein synthesis of binucleate and trinucleate pollen and tis relationship of tube emergence and growth. — PLANTA. *146,* 559—566.

LINSKENS, H. F. (1974): Fertilization in higher plants. North-Holland Publ. Co. Amsterdam.

LINSKENS, H. F. and SCHRAUWEN, J. (1969): The release of free amino acids out of the germinating pollen. — Acta Bot. Neerl. *18,* 605—614.

MASCARENHAS, J. P. (1975): The biochemistry of angiosperm pollen development. — Bot. Rev. *41,* 259—314.

PÁLFI, G. and PÁLFI, Zs. (1982): A rapid method for the determination of fertility of maize pollen with the prolineisatin reaction. — Maydica. Bergamo *27,* 107—111.

PÁLFI, G. and KÖVES, E. (1984): Determination of vitality of pollen on the basis its amino acid content. — Biochem. Physiol. Pflz. *179,* 237—240.

PÁLFI, G. and JUHÁSZ, J. (1969): Zusammenhang zwischen Wassermangel salzigem oder kaltem Wurzelmedium sowie Prolin-, und Gesamtaminosäurengehalt der Pflanzen. — Z. Pflazenaernähr. Bodenk. *124,* 36—42.

PÁLFI, G., PINTÉR, L. and PÁLFI, Zs. (1981): The proline content and fertility of the pollen of inbred maize lines. — Acta Bot. Acad. Sci. Hung. *27,* 179—187.

RAI, K. K. and STRESKOPF, N. C. (1974): Amino acid comparisons in male sterile wheat derived from *Triticum timopheevi* ZHUK. Cytoplasm and its fertile counterpart. — T.A.G. Berl. *44,* 124—127.

STANLEY, R. G. and LINSKENS, H. F. (1974): Pollenbiology, Biochemistry, Management. Springer Verlag. Berlin—Heidelberg—New York.

TUPY, J. (1964): Metabolism of proline in styles and pollen tubes of *Nicotiana alata.* In: Pollen Physiology and Fertilization (Ed. H. F. LINSKENS), pp. 86—94. North-Holland, Amsterdam.

ZHANG, H. Q. and CROES, A. F. (1983): Proline metabolism in pollen: degradation of proline during germination and early tube growth. — PLANTA, *159,* 46—49.

ZHANG, H. Q., CROES, A. F. and LINSKENS, H. F. (1982): Protein synthesis in germinating pollen of *Petunia:* role of proline. — PLANTA. *154,* 199—203.

YAMADA, N. and KONO, Y. (1976): Studies on the developmental physiology in rice pollen. — Proc. Crop. Sci. Soc. Tokyo, *45,* 279—287.

Addresses of the authors:
G. FÁLFI
Department of Plant Physiology
Attila József University
H—6701 Szeged, P.O. Box 654.
Hungary

S. GULYÁS
Department of Botany
Attila József University
H—6701 Szeged, P.O. Box 657.
Hungary