

Changes in nucleic acid and protein levels during *in vitro* germination and elongation of *Lilium longiflorum* cv. "Ace" pollen

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Abstract

While changes in nucleic acid and protein levels during germination and subsequent tube elongation have been determined for a number of pollens, they have not been extensively examined for *in vitro* grown *Lilium longiflorum*, cv. 'Ace' pollen. Nucleic acids and proteins were extracted with cold trichloroacetic acid (TCA), cold-hot TCA or cold TCA and potassium hydroxide-perchloric acid (KOH-HClO₄). Following extraction, RNA, DNA and total protein were assayed colorimetrically with orcinol, diphenylamine and Folin-Phenol reagents, respectively. Extraction of 500 x g supernatants with KOH-HClO₄ yielded less RNA than either of the TCA-extraction procedures which gave similar nucleic acids and protein recoveries. Whereas total protein levels decreased initially and then increased during 36 h, RNA and DNA levels rose throughout the time-course. Precipitation and quantitation of nucleic acids and protein from homogenized and sonicated 500 x g pellets resulted in time-dependent alterations in levels of macromolecules which differed from those for 500 x g supernatants. Whereas DNA and RNA levels increased and then decreased over 36 h, total protein levels remained constant for 12 h and then declined during the next 24 h. Addition of the data obtained for 500 x g supernatants to those for 500 x g pellets revealed that total protein levels increased 2.4 times for the first 12 h and thereafter remained constant, that RNA levels increased 9.8 times for the first 12 h and then levelled off and that the DNA content rose more than 5 times over 36 h.

INTRODUCTION

Although there are a number of reports indicating that nucleic acid and protein syntheses occur during microsporogenesis and germination of pollens and elongation of their tubes for a variety of plants (Stanley, Young, 1962; Young, Stanley, 1963; Tano, Takahashi, 1964; D'Amoto et al., 1965; Tupý, Süß, 1965; Dexheimer, 1966, 1968; Tupý, 1966; Mascarenhas, 1966; Stanley, Yee, 1966; Steffensen, 1966; Linskens, 1967; Sauter, Marquardt, 1967; Mascarenhas, Bell, 1967, 1969, 1970; Takats, 1967; Mascarenhas, Goralnick, 1971; Mascarenhas, 1965, 1971a, b; Linskens et al., 1970, 1971; La Fountain, Mascarenhas, 1971, 1972; Peddada, Mascarenhas, 1972, 1975;

Nygaard, 1973; Mascarenhas et al., 1974; Tupý et al., 1974, 1977; Charzyńska, Maleszka, 1978), the possible occurrence of nucleic acid and protein syntheses by *Lilium longiflorum* cv. 'Ace' pollen during *in vitro* germination has been examined by only a few investigators (Steffensen, 1966, 1971; Linskens, 1967) to my knowledge.

This paper reports time-dependent changes in RNA, DNA and total protein for *Lilium longiflorum* cv. 'Ace' (lily) pollen which was germinated 2, 12, and 36 h *in vitro*.

MATERIALS AND METHODS

Germination conditions

One-two g fresh weight lots of freshly harvested or stored (1 month, 4°C) *Lilium longiflorum* cv. 'Ace' pollen were sown in sterile SYB medium (Rosen et al., 1964) for 2, 12 and 36 h at $25 \pm 2^\circ\text{C}$. Germination percentages were estimated by removing drops at random and scoring the number of grains with visible tubes. Because the germination percentages varied from 60-90%, the data were normalized.

Extraction

Following germination, pollen was separated from its growth medium by centrifugation at 500 x g for 10-15 min. Ungerminated grains

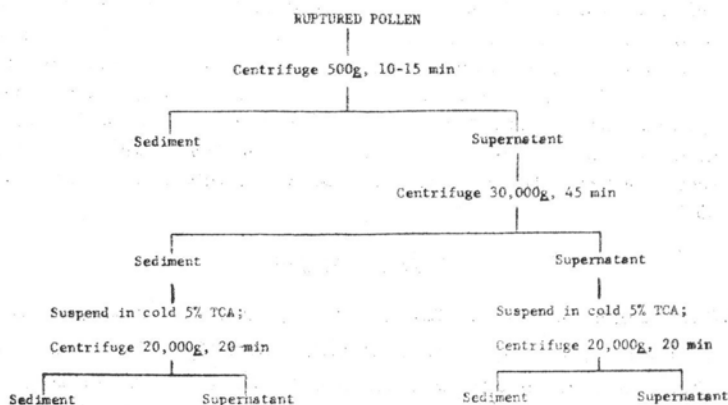


Fig 1. Summary of Method I ("high speed" centrifugation — cold TCA extraction) for the preparation of nucleic acid and protein

One-two g fresh weight lots of freshly harvested or stored (1 month, 4°C) *Lilium longiflorum* cv. 'Ace' pollen were sown in sterile SYB medium (Rosen et al., 1964) for 2, 12 and 36 h at $25 \pm 2^\circ\text{C}$; germination percentages were estimated by removing drops at random and scoring the number of grains with visible tubes; following germination, pollen was separated from its growth medium by centrifugation at 500 x g for 10-15 min; harvested pollen was ruptured by the addition of two volumes of double distilled water.

were ruptured 5 min with a Potter-Elvehjem tissue homogenizer and then an additional 5 min with an "L" and "R" Ultrasonicator. Pollen was ruptured in double distilled H_2O at a ratio of 1 g fresh weight per 20 ml H_2O . With this method cell breakages of 90% were routinely obtained as judged by microscopic observation. Grains with attached tubes were ruptured by the addition of two volumes of H_2O . Because this osmotic method of tube rupture could have resulted in incomplete release of cytoplasm from the attached grain, the pellet which resulted from a 500 x g centrifugation of the osmotically released cytoplasm was homogenized and sonicated.

To ensure the most complete recovery, three methods of extracting nucleic acids and protein were compared. These methods were: "high speed" centrifugation of homogenates followed by nucleic acids and protein precipitation with TCA (Method I, Fig. 1); treatment of homo-

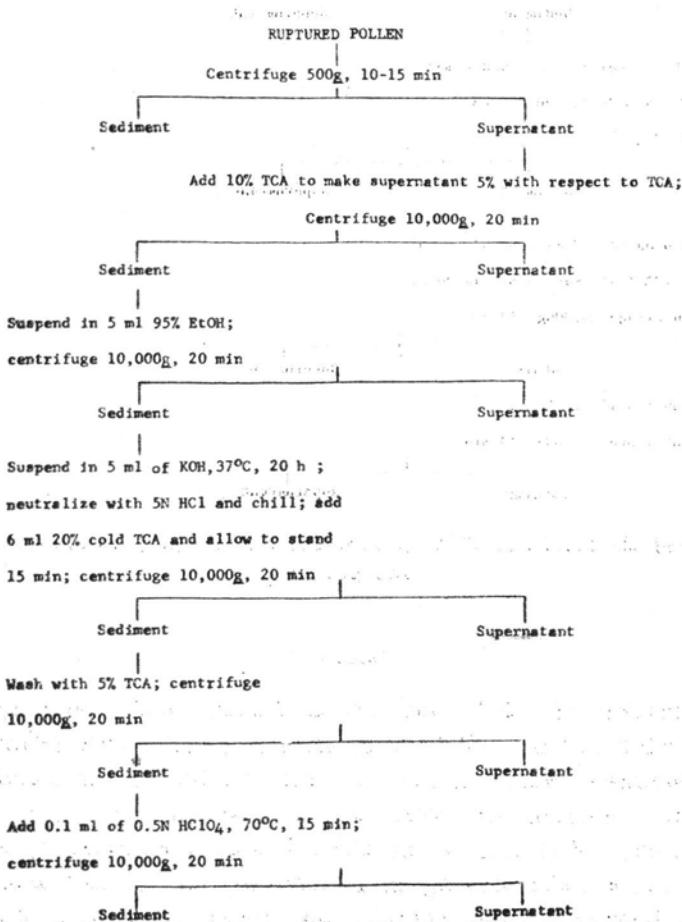


Fig. 2. Flow chart of Method II (cold TCA, KOH- $HClO_4$ extraction) for nucleic acid and protein preparation

genates with TCA followed by processing of ethanol-washed, acid-insoluble substances through a KOH-HClO₄ extraction procedure (Method II, Fig. 2), and extraction with cold-hot TCA (Method III, Fig. 3).

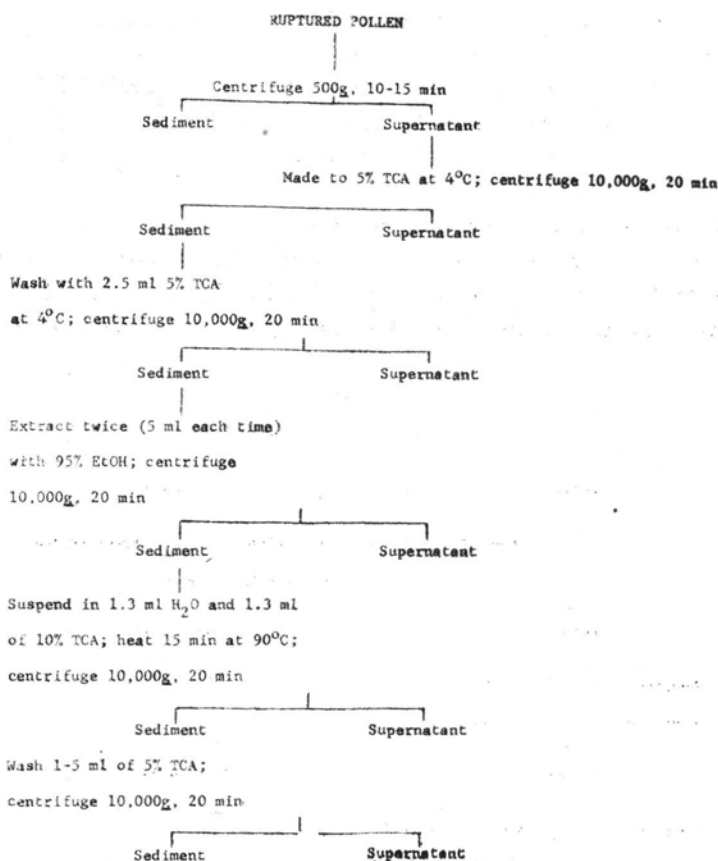


Fig. 3. Method III (cold-hot TCA) procedures for the preparation of nucleic acid and protein

Assays

Quantification of DNA was accomplished with diphenylamine at 610 nm (Schneider, 1957) with salmon sperm DNA (Pabst Laboratories, Milwaukee, Wisc.) in pH 7.2 Tris-HCl buffer for standard curve construction. Detection of RNA was at 660 nm with orcinol reagent (Schneider, 1957) and yeast RNA (Pabst Laboratories, Milwaukee, Wisc.) in 0.15 M NaCl serving as a standard. The orcinol was recrystallized from benzene. Total protein was determined with Folin-Phenol reagent (Lowry et al., 1951) with bovine serum albumin as a standard.

RESULTS

The time-dependent changes in levels of nucleic acids and protein prepared by the three methods are summarized in Fig. 4 A-C. Extrac-

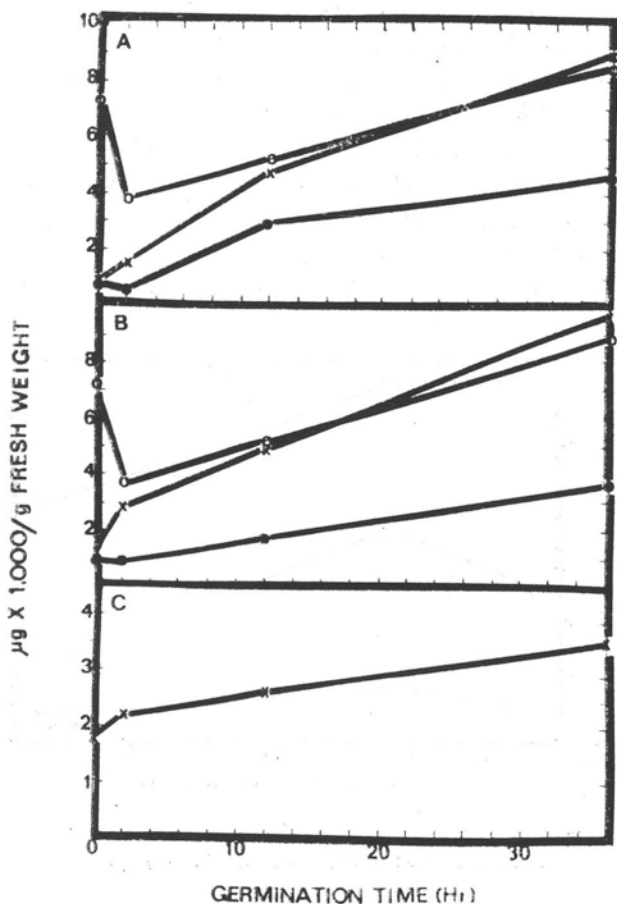


Fig. 4. A) Time-dependent changes in levels of nucleic acids and protein prepared by Method I ("high speed" centrifugation — cold TCA extraction)

Quantification of DNA was with diphenylamine at 610 nm (Schneider, 1957) with salmon sperm DNA (Pabst Laboratories, Milwaukee, Wisc.) in pH 7.2 Tris-HCl buffer for standard curve construction; detection of RNA was at 660 nm with orcinol reagent (Schneider, 1957) and yeast RNA (Pabst Laboratories) in 0.15 M NaCl serving as a standard; the orcinol was recrystallized from benzene; total protein was determined with Folin-Phenol reagent (Lowry et al., 1951) with bovine serum albumin as standard o—o = total protein, x—x = RNA, ●—● = DNA. Data are averages of at least 2 and sometimes 3-4 determinations throughout.

B) Time course for changes in nucleic acids and protein prepared by Method III (cold-hot TCA)

C) Time-related alterations in levels of nucleic acids and protein obtained by Method II (cold TCA, KOH-HClO₄ extraction)

tion with KOH-HClO₄ yielded approximately 3 times less RNA than extraction with either cold TCA or cold-hot TCA for 36 h germinated pollen. Recoveries of RNA, DNA and protein prepared by either the cold TCA or cold-hot TCA methods were quite similar. Whereas total protein levels decreased by approximately 50% during the first 2 h of germination, they more than doubled from 2 to 36 h. In contrast, RNA levels increased by a factor of 9 during a 36 h time-course. The DNA content rose 3.5 to 4.5 times over 36 h.

Fig. 5 shows the alterations in levels of DNA, RNA and total protein precipitated from the 500xg pellet which was homogenized and sonicated. The patterns of change differ markedly from those for the 500 x g supernatants. Whereas DNA levels did not markedly change from 2 to 36 h, the levels of RNA rose 1.4 times from 2 to 12 h and

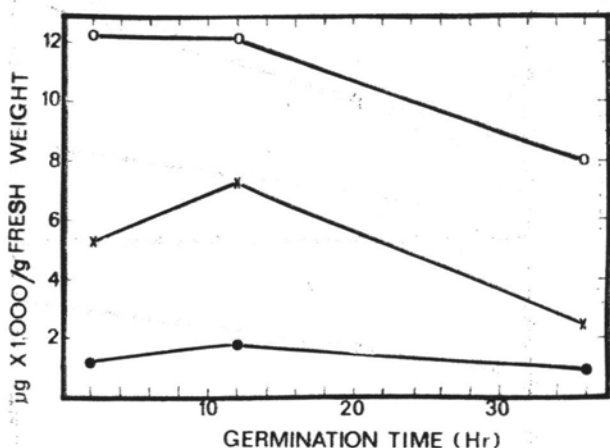


Fig. 5. Time-course for nucleic acid and protein level changes in cold-hot TCA substances extracted from a 500 x g pellet

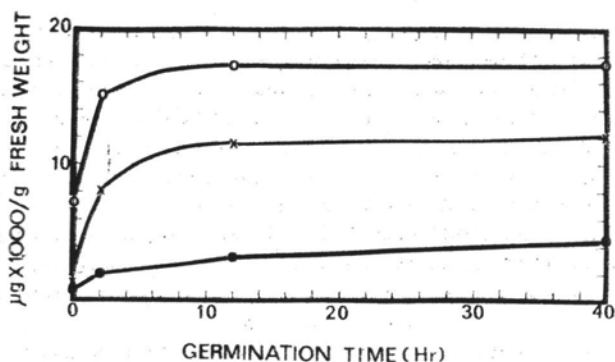


Fig. 6. Alterations in nucleic acid and protein levels in total homogenates accompanying germination and tube elongation as a function of time

then decreased 67% from 12 to 36 h. In contrast, total protein levels remained constant from 2 to 12 h and then declined 25%. When the data for the 500xg pellet (Fig. 5) were added to those for the 500xg supernatant (Fig. 4B), the graph shown in Fig. 6 resulted. Total protein levels increased 2.4 times during the initial 12 h and subsequently remained constant. Whereas RNA levels increased 9.8 times for the first 12 h and then levelled off, DNA content rose more than 5 times over 36 h.

DISCUSSION

Does RNA synthesis occur in *in vitro* germinating lily pollen?

The data presented in Fig. 6 demonstrate that orcinol-positive material increased 9.8 times between 2 and 12 h and then levelled off. This suggests that RNA synthesis does indeed occur during *in vitro* lily pollen germination and subsequent tube elongation. This suggestion is supported by the electron microscopical autoradiographic observation that elongating pollen tubes of the same *Lilium* species employed here incorporate [³H]-uridine into the growing tip of the tube and that this incorporation was sensitive to RNase (Dashek, 1966; Dashek, Rosen, 1966). The growing tip of the pollen tube contains abundant cytochemically detectable RNA (Rosen et al., 1964).

Linskens (1967) compared the ribosomal profiles of ungerminated *Lilium longiflorum* cv. 'Ace' and "Nelly White" pollen with those from pollen germinated 2 and 12 h. He observed that the ungerminated grain contained 5 peaks, the first of which was the polysome fraction which incorporated [¹⁴C]-UL-algal protein hydrolyzate. The other peaks were considered as ribosomal fractions of decreasing sedimentation coefficients. Linskens noted that the monosomal fraction decreased after 2 h of germination and that the polysomal fraction increased. However, it is possible to interpret these data as changes in the aggregation status of ribosomes rather than reflecting synthesis of RNA. In addition, RNA synthesis is known to occur in pollen tubes other than *Lilium*, e.g. *Nicotiana* (Tano, Takahashi, 1964; Tupý, Süss, 1965), *Tradescantia* (Mascarenhas, 1966, 1971a; Mascarenhas, Bell, 1967; Dexheimer, 1968; Mascarenhas, Goralnick, 1971) and *Paeonia* (Sauter, Marquardt, 1967).

The initial high rate of RNA synthesis in germinating lily pollen is consistent with that found for *Tradescantia* pollen where the rates of both RNA and protein syntheses are high during the first h of pollen

tube growth (Mascarenhas et al., 1974). Both the rates of RNA and protein syntheses decline with time in *Tradescantia* pollen as they do in germinating lily pollen. Mascarenhas et al. (1974) concluded that the types of RNA and proteins required for *Tradescantia* pollen tube elongation and generative cell division are synthesized early after germination. Tupý (1966) concluded that the RNA which occurs in pollen tubes is stable mRNA and that it is complexed with ribosomes. This conclusion was based on the resistance of *Nicotiana alata* Link et Otto pollen tubes to actinomycin D, low sensitivity toward chloramphenicol and stimulation of RNA and protein syntheses by 2-thiouracil. Earlier, Tupý and Süß (1965) stated that the actual amount of DNA-dependent synthesis of RNA represents a minute fraction of the total RNA synthesized. However, La Fleur and Mascarenhas (1973) concluded that the RNA synthesized during *Tradescantia poludosa* L. pollen germination and tube growth is probably mRNA. This conclusion is based on the observations that ribosomal and transfer RNA do not appear to be synthesized in pollen tubes in general (Tano, Takahashi, 1964; Steffensen, 1966; Mascarenhas, Bel, 1970). Tano and Takahashi found that the small amount of RNA synthesized in *Nicotiana* pollen following 7 h of germination was probably mRNA. Recently, Tupý et al. (1977) presented evidence for ribosomal RNA (5S, 18S, and 28S) synthesis in cultures of *Nicotiana tabacum* L. pollen tubes. A higher rate of 5S RNA synthesis with respect to 18S plus 28S RNA was observed. The transcription of 5S RNA genes decreases during *Tradescantia* pollen germination (Mascarenhas, Goralnick, 1971).

Does *in vitro* germinating lily pollen synthesize DNA?

The data shown in Fig. 6 indicate that the DNA content of germinating pollen increased more than 5 times suggesting that *in vitro* grown lily pollen can synthesize DNA. While a doubling of the DNA could be explained by division of the generative nucleus to form the two sperms, the second increase in DNA is more difficult to interpret. In this connection, Stanley and Young (1962) demonstrated that [³H]-thymidine was incorporated into the tube nucleus, thus, raising the possibility that it is capable of DNA synthesis. The synthetic capability of the tube nucleus was also demonstrated by Mascarenhas (1965) who observed that it can synthesize RNA in *Tradescantia* pollen tubes. To determine whether the tube nucleus of germinating lily pollen can synthesize DNA, cytophotometric analyses of Feulgen-positive tube nuclear material could be quantitated.

Are proteins synthesized during lily pollen germination and tube elongation *in vitro*?

The increase in total protein suggests that protein synthesis does occur during *in vitro* lily pollen germination and subsequent tube elongation. This suggestion is supported by the observation that the incorporation of [¹⁴C]-proline into TCA-precipitable cytoplasm increases with time (Dashek et al., 1971; Dashek, Harwood, 1974). Furthermore, Linskens (1967) observed that ribosomes isolated from germinating *Lilium longiflorum* cv. 'Ace' pollen can incorporate [¹⁴C]-algal protein hydrolyzate. This incorporation was most intense during the first few h of germination and was reduced 10 h after germination when pollen tubes ceased to grow. It is also supported by the demonstration of protein synthesis in *Pinus* (Stanley et al., 1958) *Nicotiana* (Tupý, 1966), *Petunia* (Linskens et al., 1970) and *Tradescantia* (Mascarenhas, Bell, 1969) pollen tubes. These reports are to be contrasted to that of Stanley and Linskens (1964) who demonstrated enzyme activation in germinating *Petunia* pollen.

Whether there are different classes of proteins synthesized during lily pollen germination and tube elongation remain to be explained. This could be accomplished by polyacrylamide gel electrophoresis of proteins extracted from ungerminated pollen and those from pollen germinated for various periods of time.

In this connection, an SDS acrylamide gel electrophoretic analysis of proteins derived from *Tradescantia* pollen tubes labeled continuously for periods of 1-6 h with a mixture of [¹⁴C]-amino acids indicated a banding pattern almost identical for all periods of labeling except for one band which disappeared after 1 or 2 h of the elongation (Mascarenhas et al., 1974).

What are the limitations of the present work?

The possibility that at least a portion of the increases in nucleic acids and total protein which accompanied germination and tube elongation was due to bacterial and fungal contamination cannot be entirely excluded since the pollen, unlike the culture medium, was not sterile. However, although an aliquot of the medium was not plated out, the medium did not show visual indications of contamination. One control would be to incubate sterile medium for 35 h in the absence of pollen followed by attempted extraction and quantitation of nucleic acids and protein from the medium. The time-dependent plateauing of the RNA and protein levels (Fig. 6) can be used as one indicator that the RNA increases which accompany lily pollen germination and tube elongation

are not due to bacterial contamination. Because lily pollen contains polysaccharides which are composed of pentoses (van der Woude et al., 1971) that could conceivably contaminate the TCA-precipitable RNA (e.g., a polysaccharide containing an attached uridine phosphate molecule can be extracted with phenol (Mascarenhas, 1971a)) and because the orcinol procedure assays for pentoses the possibility exists that the values reported here for RNA are not accurate.

Although RNA was quantitated by two colorimetric procedures, DNA and protein levels were each detected by only one colorimetric assay. Thus, to definitively establish that *in vitro* germinating lily pollen can synthesize nucleic acids and protein, time-dependent changes in the incorporation of [^{14}C]-leucine into extracted and purified RNA, DNA and total protein, respectively, should be determined.

Finally, although averages rather than means and standard deviations are presented because of duplicate rather than triplicate repetitions, the close agreement between the data for the duplicates and in Fig. 4A and Fig. 4B lend credence to the validity of the data.

Acknowledgements

Appreciation is extended to Ms. Patty Straight for clerical assistance. Supported in part by a travel award from the Department of Biology, West Virginia University.

Appendix

Following the preparation of this manuscript, a paper (Süss, Tupy, 1979) appeared which demonstrated the presence of RNA in a pollen tube cell wall fraction derived from germinating *Nicotiana tabacum* L. pollen. In addition, a paper has appeared (Singh, Malik, 1979) which indicates that RNA and protein syntheses occur during *Amaryllis vittata* pollen germination and tube elongation.

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