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RNA, Proteins and Polyamines During Tube Growth in Germinating Apple Pollen[1]

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ABSTRACT

Variations of RNA, protein, and free- and trichloroacetic acid-soluble bound polyamine levels were determined during tube growth in germinating *Malus domestica* Borkh. cv. Starkrimson pollen.

During rehydration of pollen no marked differences were observed, whereas, during germination, RNA, proteins, and polyamines showed parallel decreases. At the same time, there was synthesis of RNA and polyamines as indicated by use of labeled precursors. The data indicate that during germination: (a) the genes for rRNA, tRNA, and probably mRNA are active; (b) the enzymes involved in polyamine biosynthesis are very active. High levels of free arginine during the first 15 minutes were observed, probably in response to a demand for this precursor in polyamine biosynthesis. Moreover, profiles of the variations in the specific activities of RNA and polyamines showed similar patterns. The results indicate that biosynthesis of RNA and polyamines precedes tube emergence. The possible role of these compounds, which are known to be released into the medium in the progamic phase of the fertilization processes, is considered.

There are numerous reports on metabolic processes involved in the germination of different kinds of pollen, *e.g.* pollen of *Tradescantia*, *Petunia*, *Lilium*, *Nicotiana* etc. (20, 26, 30). In contrast, there is a lack of information concerning pollen grains of fruit trees in this regard. Pollen germination is a complex process that involves many physiological aspects such as gamete differentiation (26) and cell-cell recognition during fertilization (6, 7).

The definitive inactivation of genes of rRNA which seems to take place before pollen grain maturation, and the possibility that the initial tube development occurs without RNA synthesis, has been a source of controversy (20, 34). In a number of biological systems, nucleic acids are known to interact with polyamines which are involved in different cellular events such as growth, differentiation, and division (5, 31). In pollen, little attention has been focused on the presence and functions of polyamines (2, 14). In particular, nothing is known about the role of polyamines in the emergence and growth of the tube during germination. The results reported here concern the changes in concentration of polyamines, RNA, and proteins during tube growth in apple pollen germination *in vitro*.

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MATERIALS AND METHODS

Pollen and Culture Conditions. Samples of mature pollen of *Malus domestica* Borkh. cv. Starkrimson were obtained (April 1978) from plants grown in experimental plots of Defendi Farm, Altedo (Bologna), collected as previously described (4) and stored in glass containers at −20 C in the presence of NaOH pellets. Under such conditions viability (9) and germination (4) were higher than 90% even 2 years after harvest. Germination was preceded by rehydration of the pollen in humid air (RH 100%) for 30 min at 30 C. The germination of pollen was studied by mass culture carried out in flasks, in which 100 mg pollen were suspended in 50 ml germination medium (4) and shaken (80 cycles/min). After 2 h at 30 C in the dark, the tubes burst. For the determination of polyamines and arginine, 40 mg pollen in 20 ml medium were used. For protein and nucleic acid determination 20 mg in 10 ml and 200 mg in 100 ml, respectively, were grown in flasks on a shaker. All determinations are referred to the initial weight of pollen taken directly from storage at −20 C, before rehydration.

Amine Analysis. Polyamines were extracted in 5% (w/v) TCA or 5% TCA (w/v) −0.1 N HCl in a Potter-Elvehjem homogenizer, and analyzed by two procedures: (a) direct dansylation (24), and (b) automatic ion-exchange column chromatography (1). According to (a), polyamines were separated on TLC precoated plates of Silicagel 60 with concentrating zone (Merck). The spots were scraped from the plates, extracted in pure acetone in a vortex mixer, and centrifuged; the fluorescence was measured with a Jasco FP-550 spectrofluorimeter (excitation 360 nm, emission 506 nm). Arginine was also analyzed by method (b). The presence of bound polyamines was investigated by analysis of the acid-hydrolyzed soluble 5% TCA extract. Samples were hydrolyzed in 6 *N* HCl at 110 C for 24 h (35).

Proteins. Proteins were extracted from pollen after collection on a Millipore filter followed by washing and immersion of the pollen in 1 N NaOH; in some experiments, after rehydration, proteins were extracted by direct immersion of pollen in NaOH solution. Protein concentration was determined according to Lowry *et al.* (16).

Nucleic Acids. RNA was extracted as previously described (22) in a Potter-Elvehjem homogenizer and determined by the method of Kirby (12) as modified by Li and Andersen (13). Nucleic acids were separated on a Sephadex G-100 column (Pharmacia Fine Chemicals) (106 × 1 cm) eluted with 20 mM potassium acetate at 4 C and detected by *A* at 260 nm.

Use of Radioisotopes. [5-^{3}H]uridine (28 Ci/mmol) and L-[5-^{3}H]arginine (16 Ci/mmol) were obtained from Radiochemical Centre, Amersham, U.K. Two hundred μCi uridine or 9.37 μCi of arginine were added to the medium of each sample at the beginning of the experiments. The radioactivity was measured in a

liquid scintillation counter Isocap/300 (Nuclear Chicago).

Respiration Measurements. Respiration of pollen during germination was measured by the polarographic O_2 electrode technique (8) in the presence or absence of 2 mM KCN and 0.2 nM antimycin.

RESULTS AND DISCUSSION

Rehydration and Germination. The rehydration was found to be necessary for a consistent response of the pollen grains. During rehydration, no marked differences in the concentration of RNA, polyamines, and proteins were observed as shown in Figure 1. The unchanged amount of total RNA should indicate that neither synthesis nor breakdown had occurred during rehydration. Some proteins are immediately removed from the exine when the pollen is washed during the collection on the filter (Fig. 1).

Figure 2 shows the percentage of germination and Figure 3 the per cent frequencies of classes of different length tubes in a typical experiment of 2 h duration. Respiration in germinating pollen is cyanide- and antimycin-sensitive, indicating that it is aerobic.

Proteins and RNA During Germination. During the progress of germination, the total amounts of proteins, RNA, and polyamines exhibit similar decreases (Fig. 1). Proteins are known to be released from the pollen into the medium as recently shown for apple pollen grown under the same experimental conditions (25). The amount of total RNA found in dry apple pollen is very similar to that found by Süss and Tupý (28) in *Nicotiana alata* pollen, where it appears to remain practically constant during the first 6 h of germination. The progressive decrease in the total amount of RNA found in apple pollen has also been observed in other pollen (33) and was attributed to a progressive release of RNA from pollen tubes into the culture medium. This has been verified for apple pollen (25). In addition, an increase in ribonuclease and protease activities might occur as a consequence of a decrease in the polyamine content, as suggested by others (10, 11). We have observed that the most marked decrease takes place within the first 30 min of germination and is restricted to rRNA and tRNA; 5S RNA is present in very low concentration (Fig. 4). On the other hand, another type of RNA, a "fourth RNA," eluted after tRNA, shows a marked increase in amount within 30 min as compared with its content in ungerminated pollen (Fig. 4).

The experiments with [^{3}H]uridine (Fig. 5) show that the "fourth RNA" is characterized by its very rapid incorporation of [^{3}H]uridine as compared with other RNA. The maximum uridine incorporation, which is achieved after about 20 min of germination (Fig. 5, insert), accounts for nearly 60% of the radioactivity incorporated into all the nucleic acids. This incorporation decreases sharply, while the other RNA continue to incorporate label throughout the 2 h of pollen germination. A similar behavior of the "fourth peak" from the Sephadex G-100 columns, which has been tentatively identified as poly(A)RNA, has been found for *Helianthus tuberosus* (23). In this tissue, the transition from dormancy to the active state is characterized by a sudden rise followed by a decrease in this RNA. Recently, Süss and Tupý (30) have observed in germinating *Nicotiana tabacum* pollen an early synthesis of poly(A)$^+$ mRNA followed by a decline and an increase in the concentration of poly(A)$^-$ mRNA; moreover, in pollen of other species, an early synthesis of a heterodisperse RNA has been reported to occur during germination (18, 19). Assemblage of stored mRNA with preexisting ribosomes has been observed during the first minutes of the germination in nonrehydrated pollen, *e.g.* in *Nicotiana tabacum* the percentage of polysomes increased from 12 to 46% (32) and a rise of the same order occurred also in *Petunia* (15) and in *Tradescantia* (17). In contrast to the results reported by others for *Tradescantia* (18, 19) and *Lilium* (27), our present data for apple and those for *N. tabacum* (29, 30, 32, 34) suggest that during germination of the pollen of

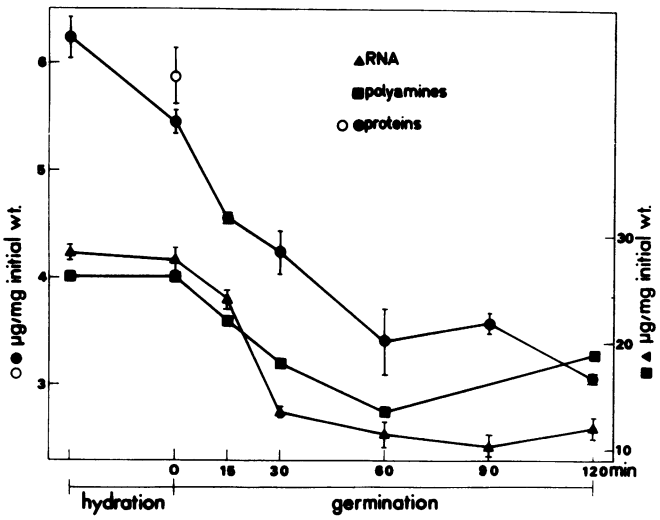

FIG. 1. Changes in RNA, proteins, and free polyamines during rehydration and germination of apple pollen. ○, Protein determination without washing of pollen; ●, protein determination with prewashing of pollen.

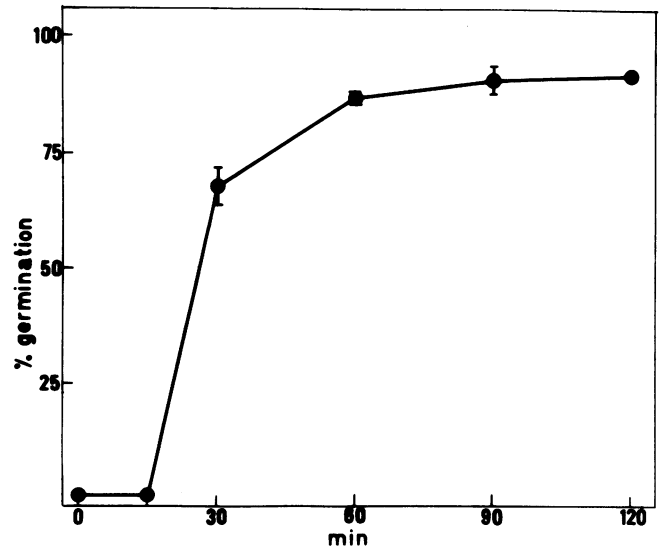

FIG. 2. Germination of apple pollen.

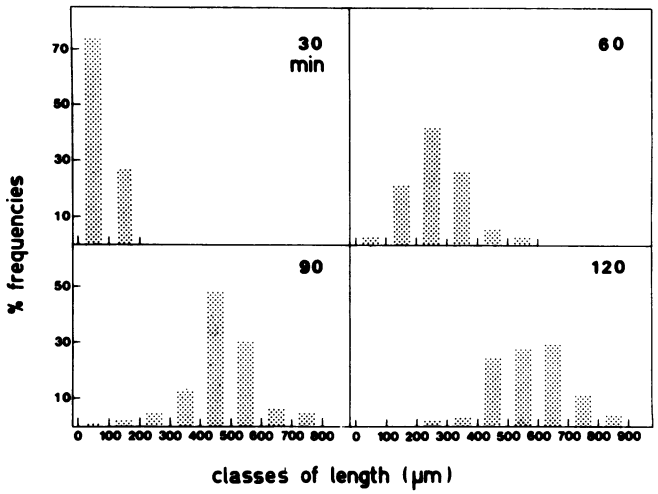

FIG. 3. Frequencies (in per cent) of classes of tube length of apple pollen during germination.

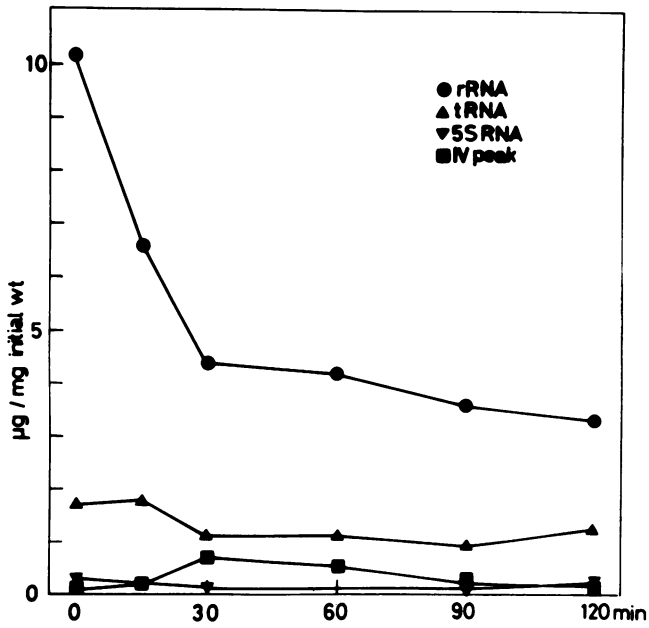

FIG. 4. Changes in rRNA, 5S RNA, tRNA, and IV peak ("fourth RNA") during germination of apple pollen.

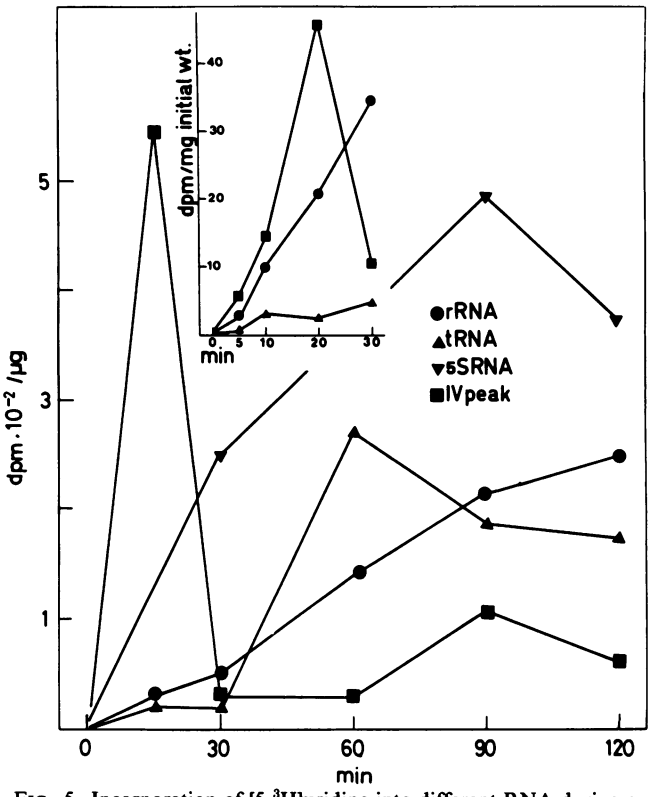

FIG. 5. Incorporation of [5-^{3}H]uridine into different RNA during germination of apple pollen.

these species not only the genes for mRNA but also those for rRNA and tRNA are active.

Polyamines. Figure 6, A and B show the amounts of free and TCA-soluble bound polyamines and arginine detected during hydration and germination by automatic ion-exchange column chromatographic analysis (1). Both methods of analysis gave similar results. Only putrescine, spermidine, and spermine were detected in apple pollen.

Free arginine increased during the first 15 min of germination,

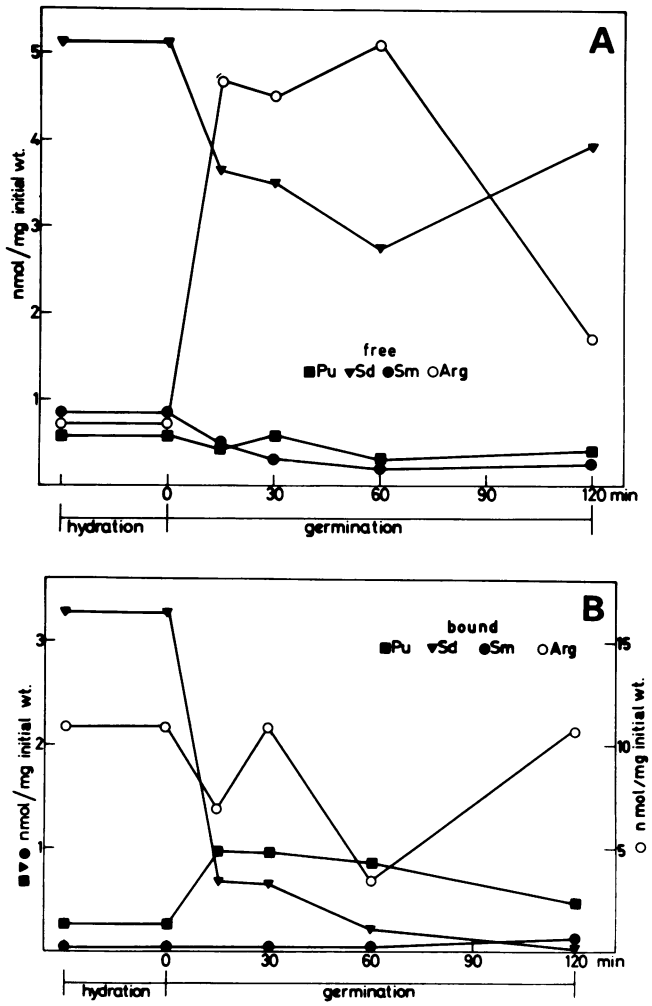

FIG. 6. Changes in (A) free and (B) TCA-soluble bound polyamines and arginine during rehydration and germination of apple pollen.

while TCA-soluble bound arginine decreased; after 2 h both reached approximately their initial value. Free and bound spermidine declined rapidly during the first 15 min. Then, while bound spermidine declined slowly, free spermidine started to increase again after 60 min. Free putrescine and spermine did not change significantly throughout the 2 h of germination. Labeling experiments with [^{3}H]arginine demonstrated (Fig. 7) an active synthesis of polyamines during the first 15 min. Moreover, free spermidine and total free polyamines exhibited a profile opposite to that of free arginine; these results could indicate a product-precursor relationship.

The transition from dormancy to germination of pollen is characterized by a sudden rise in both transcription (Fig. 5) and translation (20). As polyamines are known to promote both processes (5, 31) a requirement for them is to be expected. As already shown (Fig. 7) an active synthesis of polyamines takes place during the early stages of tube formation and growth.

Profiles of the variations in specific activity of polyamine and RNA, particularly "fourth RNA," were similar (Figs. 5 and 7).

The occurrence of bound putrescine during germination, and especially of a great amount of bound spermidine present in ungerminated pollen, raises the question of their physiological significance. Peptides conjugated with polyamines have been found in various organisms (21). On the other hand, acetylated polyamine derivatives are known to be present in most biological systems, yet, to the best of our knowledge, these compounds have not been reported to be present in plants. Acetylation and deace-

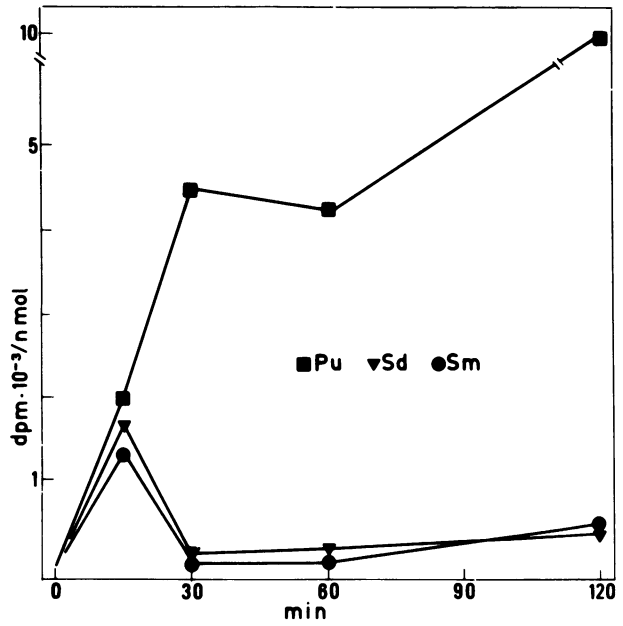

FIG. 7. Incorporation of [5-^{3}H]arginine into different polyamines during germination of apple pollen.

tylation reactions could regulate polyamine functions by affecting the binding and interaction of polyamines with nucleic acids (3).

The total pool of free and bound polyamines as well as their ratio may change during germination as a result of release into the culture medium. In fact, free polyamines were present in increasing amounts in the medium throughout the 2 h of germination under the same experimental conditions (25). The release of polyamines was detectable after 20 min of germination. Free spermidine in the medium was, in general, less than 5% of intracellular free spermidine. The release of putrescine was more than that of spermidine, and reached the intracellular putrescine level, after 2 h of germination. On the contrary, a great amount of free spermine was released into the medium at the beginning of tube emergence (30 min). Whereas the intracellular level always remained low, free spermine, after 2 h of germination, had reached more than six times this level. The release of polyamines and of RNA may suggest a possible role of these compounds in the progamic phase of the fertilization process.

In conclusion, our results indicate that the metabolism of polyamines, proteins, and RNA is not significantly affected during rehydration, but undergoes a very rapid and marked modification when the pollen is in contact with the germination medium. Our data indicate that polyamine and RNA biosynthesis precedes germination of the pollen. In fact, during the first 15 min, active synthesis of RNA and polyamine takes place, while no emergence of pollen tubes is observed (Figs. 2, 5, and 7). At the same time, progressive increases begin in proteins, polyamines, and RNA in the medium (25). This may account, at least in part, for the observed decreases in accumulation of these substances (Fig. 1). The emergence of tubes then starts and 70% of the pollen germinates within 30 min (Figs. 2 and 3). If the observed early increase in the release of substances by pollen into the medium occurs *in vivo*, it could function as an important physiological signal in the metabolic process related to tube growth during germination. Possible relationship of such a signal to fertility or sterility should be kept in mind.

LITERATURE CITED

1. ADLAKHA RC, VR VILLANUEVA 1980 Automated ion exchange chromatographic analysis of usual and unusual natural polyamines. J Chromatogr 187: 442–446
2. BAGNI N, D SERAFINI-FRACASSINI, VR VILLANUEVA, RC ADLAKHA 1978 Contenuto in poliammine, etanolammina e arginina nel polline di melo. Ortoflorofrutticoltura Ital 5: 470–476
3. BLANKENSHIP J, T WALLE 1978 *In vitro* studies of enzymatic synthesis and metabolism of *N*-acetylated polyamines. *In* RA Campbell, DR Morris, D Bartos, GD Daves, F Bartos, eds, Advances in Polyamine Research, Vol 2. Raven Press, New York, pp 97–110
4. CALZONI GL, A SPERANZA, N BAGNI 1979 *In vitro* germination of apple pollen. Sci Hortic 10: 49–55
5. COHEN SS 1978 The functions of the polyamines. *In* RA Campbell, DR Morris, D Bartos, GV Daves, F Bartos, eds, Advances in Polyamine Research, Vol 1. Raven Press, New York, pp 1–10
6. CURTIS ASG 1978 Cell-Cell Recognition. Symposium 32 of the Society for Experimental Biology. Cambridge University Press, Cambridge
7. DE NETTANCOURT D 1977 Incompatibility in Angiosperms. Springer-Verlag, Berlin
8. ESTABROOK RW 1967 Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. Methods Enzymol 10: 41–47
9. HOEKSTRA FA, J BRUINSMA 1975 Respiration and vitality of binucleate and trinucleate pollen. Physiol Plant 34: 221–225
10. KAUR-SAWHNEY R, A ALTMAN, AW GALSTON 1978 Dual mechanism in polyamine-mediated control of ribonuclease activity in oat leaf protoplasts. Plant Physiol 62: 158–160
11. KAUR-SAWHNEY R, AW GALSTON 1979 Interaction of polyamines and light on biochemical processes involved in leaf senescence. Plant Cell Environ 2: 189–196
12. KIRBY KS 1964 Isolation and fractionation of nucleic acids. *In* JN Davidson, VE Cohn, eds, Progress in Nucleic Acid Research and Molecular Biology, Vol 3. Academic Press, New York, pp 1–31
13. LI PH, WR ANDERSEN 1967 Large amounts of ribosomal RNA in colchicine-induced tetraploids of *Solanum tuberosus* L. Nature 214: 86–87
14. LINSKENS HF, ASL KOCHUYT, A SO 1968 Regulation der Nucleinsäuren-synthese durch Polyamine in keimenden Pollen von *Petunia*. Planta 82: 111–122
15. LINSKENS HF, JAM SCHRAUWEN, RNH KONINGS 1970 Cell-free protein synthesis with polysomes from germinating *Petunia* pollen grains. Planta 90: 153–162
16. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
17. MASCARENHAS JP, E BELL 1969 Protein synthesis during germination of pollen: studies on polyribosome formation. Biochim Biophys Acta 179: 199–203
18. MASCARENHAS JP, E BELL 1970 RNA synthesis during development of the male gametophyte of *Tradescantia*. Develop Biol 21: 475–490
19. MASCARENHAS JP, RD GORALNICK 1971 Synthesis of small molecular weight RNA in the pollen tube of *Tradescantia paludosa*. Biochim Biophys Acta 240: 56–61
20. MASCARENHAS JP 1975 The biochemistry of angiosperm pollen development. Bot Rev 41: 259–314
21. SEALE TW, W CHAN, J SHULKE, OM RENNERT 1979 A polyamine-conjugated peptide isolated from human plasma. Arch Biochem Biophys 198: 164–174
22. SERAFINI-FRACASSINI D, N BAGNI, GL CALZONI, A SPERANZA 1978 RNA nel polline di melo. Ortoflorofrutticoltura Ital 5: 669–673
23. SERAFINI-FRACASSINI D, N BAGNI, PG CIONINI, A BENNICI 1980 Polyamines and nucleic acids during the first cell cycle of *Helianthus tuberosus* after the dormancy break. Planta 148: 332–337
24. SMITH TA, GR BEST 1977 Polyamine in barley seedlings. Phytochemistry 16: 841–843
25. SPERANZA A, GL CALZONI 1980 Compounds released from incompatible apple pollen during *in vitro* germination. Z Pflanzenphysiol 97: 95–102
26. STANLEY RG, HF LINSKENS 1974 Pollen, Biology Biochemistry Management. Springer-Verlag, Berlin
27. STEFFENSEN DM 1966 Synthesis of ribosomal RNA during growth and division in *Lilium*. Exp Cell Res 44: 1–12
28. SÜSS J, J TUPÝ 1976 The effect of 2-thiouracil on RNA synthesis in pollen tubes of *Nicotiana alata*. Biol Plant 18: 119–125
29. SÜSS J, J TUPÝ 1978 tRNA synthesis in germinating pollen. Biol Plant 20: 70–72
30. SÜSS J, J TUPÝ 1979 Poly(A)$^+$ RNA synthesis in germinating pollen of *Nicotiana tabacum* L. Biol Plant 21: 365–371
31. TABOR CW, H TABOR 1976 1,4-Diaminobutane (putrescine), spermidine and spermine. Annu Rev Biochem 45: 285–306
32. TUPÝ J 1977 RNA synthesis and polysome formation in pollen tubes. Biol Plant 19: 300–308
33. TUPÝ J, E HRABĚTOVÀ, V BALATKOVÀ 1974 The release of RNA from pollen tubes. *In* HF Linskens, ed, Fertilization in Higher Plants. North-Holland Publ Co, Amsterdam, pp 145–160
34. TUPÝ J, E HRABĚTOVÀ, V BALATKOVÀ 1977 Evidence for ribosomal RNA synthesis in pollen tubes in culture. Biol Plant 19: 226–230
35. VILLANUEVA VR, RC ADLAKHA, R CALVAYRAC 1980 Cell cycle related changes in polyamines in *Euglena*. Phytochemistry 19: 962–964