

Auxin modification of the incompatibility response in *Theobroma cacao*

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The time course and control of floral abscission and fruit set in *Theobroma cacao* were studied after spray application of growth regulators. 1-Naphthaleneacetic acid (NAA) prevented flower abscission in a concentration dependent manner and induced the early stages of fruit development. The cytokinin benzylaminopurine (BAP) counteracted NAA but resulted in

longer fruit retention. Measurements of endogenous levels of indole-3-acetic acid showed an inverse correlation between the number of flowers per plant and auxin content. The results suggest that the genetic control of self-incompatibility in *T. cacao* may be modulated by the hormonal content of the flower.

Introduction

Self-incompatibility (SI) in flowering plants represents a biochemical recognition and rejection process that minimizes self-fertilization. Most SI plants prevent fertilization by arresting the growth of the pollen tube on the stigma or in the style of self-pollen or pollen from a close genetic relative (Dickinson and Lewis 1975, deNettancourt 1977 and references therein). SI in *Theobroma cacao* is more complex. First reported by Pound (1932), investigations into the mechanism of SI in cacao by Knight and Rogers (1953, 1955) and Cope (1958, 1959, 1962a,b) indicated that incompatible pollination did not result in inhibition of pollen germination or reduced pollen tube growth rates through the hollow style (Knight and Rogers 1955, Bouharmont 1960, Cope 1962a,b). Following self-pollination, Cope (1958) observed that sperm and egg failed to fuse in a percentage of ovules. However, despite fertilization of some ovules, flower abortion ensued. The *Theobroma* type of SI may result from the expression of the s-gene only after the haploid pollen tube and the ovules have come into contact, and the rejection results in the abscission of the entire flower. This concept suggests that s-gene expression occurs at the pollen tube-ovule interface. Thus, *Theobroma* may represent an example of an ovarian SI system, unlike the majority of SI plants (deNettancourt 1977). Recent studies of *Theobroma* (Aneja et al. 1992, 1994, Baker et al. 1997) suggest that flower abscission because of incompatible pollination

is initiated prior to the pollen tubes reaching the ovules. Aneja et al. (1992, 1994) found that pollen grains of the self-incompatible clone IMC 30 did not germinate in low pCO₂ and that elevated CO₂ could overcome SI in self-pollinated flowers. The large number of *Theobroma* clones prompted Warren (1994) and Warren and Sunita (1995) to search for predictors of SI. Despite the lack of a causal link between the expression of enzymatic activity and incompatibility, they found that enzymes such as isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), and acid phosphatase (AP) were indicators of SI. The mentor effect, for example, overcoming SI by mixing compatible with incompatible (self) pollen (Lanaud et al. 1987, Glendinning 1960, Opeke and Jacob 1967), implies that in *Theobroma* some substance other than CO₂ may condition the maternal plant. Our previous work on hormonal changes after compatible and incompatible pollination (Baker et al. 1997) indicated a strong increase in auxin after compatible pollination and a strong increase in ethylene after incompatible pollination. This hormonal response occurs prior to pollen tube-ovule interaction and suggested an incompatibility system that is influenced by auxin. The oppression of abscission by CO₂ (Aneja et al. 1994) and subsequent self-compatibility may result from minimizing ethylene effects. It is conceivable that higher auxin levels also counteract the abscission signal and therefore auxin may affect or control the self-in-

Abbreviations – ABA, abscisic acid; ACC, aminocyclopropane-1-carboxylic acid hydrochloride; AVG, 2-aminoethoxyvinyl-glycine; BAP, benzylaminopurine hydrochloride; NAA, 1-naphthaleneacetic acid; STS, silverthiosulfate.

compatibility response in *Theobroma*. The purpose of this study was to determine the effect of modified phytohormone content on the incompatibility reaction and fruit set in *Theobroma cacao*.

Materials and methods

All research was conducted at the Clones and Hybrids section of the Almirante Cacao Research Station in Itajuípe, Bahia, Brazil in February 1998 and June 1999. The experiments were conducted on *Theobroma cacao* L. clone TSH-565 except where noted otherwise.

Pollination

The day prior to anthesis, flower buds were bagged with mosquito netting to prevent pollinator access. The next day, unopened buds were removed and at least 30 flowers per

treatment from two to four trees were hand-pollinated. Incompatible pollinations were done with flowers from the same tree and compatible pollinations with pollen from the clone CC10. Immediately after pollination, test solutions were applied and mosquito netting was placed over the pollinated flowers and secured to prevent pollinator access. After pollination, the fate of the flowers was monitored for 2 weeks and the number of remaining flowers and developing fruits was counted daily.

Application of growth regulators

Stock solutions (10 mM) of 1-naphthaleneacetic acid (NAA, K-salt, Sigma # N1145, St. Louis, MO, USA) and benzylaminopurine hydrochloride (BAP, Sigma # B5920), abscisic acid (ABA, Sigma # A 7383), aminocyclopropane-1-carboxylic acid hydrochloride (ACC, Sigma # A0430) and (2-aminoethoxyvinyl)-glycine (AVG, Sigma # A1284) were prepared in distilled water and diluted to 10^{-3} to 10^{-5} M. Silverthiosulfate (STS) was prepared by mixing 100 mM AgNO_3 with 400 mM $\text{Na}_2\text{S}_2\text{O}_3$ and then diluted to 1:20. The compounds were spray-applied in a carrier solution of 0.01% Tween-20 to individual flowers or flower clusters either before pollination and/or after pollination for pre-determined times.

Hormone analysis

Unopened flowers (1–2 days prior to anthesis) were collected from various clones and analyzed for IAA and abscisic acid. Because of the limited number of flowers from some genotypes, flowers were pooled, frozen and lyophilized. The extraction and purification method for IAA and ABA and their quantification by gas chromatography with selected ion monitoring mass spectroscopy (GC-SIMS) were as described earlier (Baker et al. 1997). The results are reported as the mean of 3 replicates \pm SD.

Microscopy

Flowers were collected in vials filled with 2% glutaraldehyde in 10 mM phosphate buffer, pH 7. The gynoecia were prepared following the method of Martin (1959) and stored until sectioning could be performed. Thick Vibratome sections (40–50 μm) were examined under an epifluorescence microscope (Zeiss Axiophot, Thornwood, NY, USA).

Results

Floral abscission after application of phytohormone

Theobroma flowers are typically short-lived and abscise within 48 h. To examine whether the application of phytohormones affects the retention, flowers remaining on trees were counted and expressed as percentage of the initial number of flowers (Fig. 1A). The application of 10^{-5} M ABA to unpollinated flowers prolonged flower viability such that about 15–20% of the flowers were retained for about 1 week. The delay in abscission after ABA treatment was limited to unpollinated flowers. Incompatible pollination

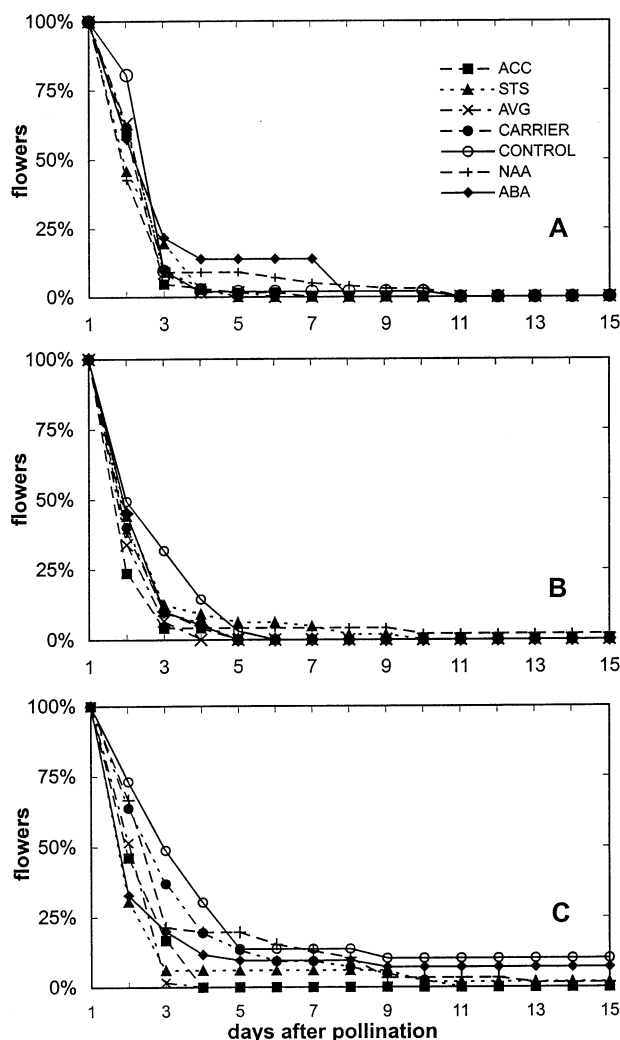


Fig. 1. The time course of floral abscission in *T. cacao* in unpollinated (A) flowers and after compatible (B) and incompatible (C) pollination. Application of 10^{-5} M ABA, ACC, STS, AVG, NAA or carrier (0.01% Tween-20, control) resulted in a similar abscission pattern.

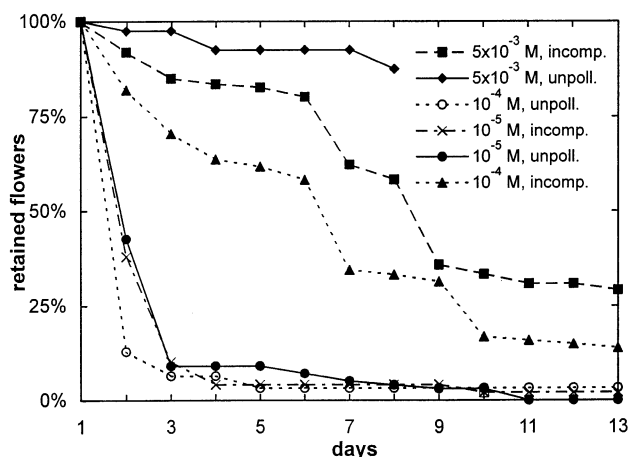


Fig. 2. The flower retention after pollination and spray application of NAA from 10^{-5} to 5×10^{-3} M was concentration dependent. NAA caused a substantial retention even of unpollinated flowers, presumably by preventing the development of the abscission layer.

delayed abscissions compared with all other treatments suggesting that pollination, regardless of type, constituted a positive, flower-retaining signal (Fig. 1B). After compatible pollination, about 10–15% of the control flowers were retained, followed by ABA-treated flowers, indicating that elevated ABA levels do not necessarily induce abscission but possibly confer a degree of robustness. NAA application to unpollinated flowers showed an early effect of flower retention. All other treatments had no effect.

Concentration of NAA

Varying the concentration of NAA in the spray solution showed a dramatic effect on flower retention (Fig. 2). While 10^{-5} M NAA showed no measurable effect in pollinated flowers (Fig. 1), 10^{-4} M NAA improved flower retention in incompatibly pollinated flowers and 50% of the flowers were still attached 1 week after pollination. Higher NAA concentrations (5×10^{-3} M) significantly improved flower retention in both incompatibly pollinated and unpollinated flowers. A single application prevented abscission but was insufficient to prevent wilting of the flowers. Despite wilting NAA-treated flowers did not abscise.

Timing of NAA application

As NAA had a dramatic effect on flower retention, it was important to verify the most efficient time for elevating the auxin content in the tissue. When NAA was applied prior to anthesis, the retention of flowers was markedly improved

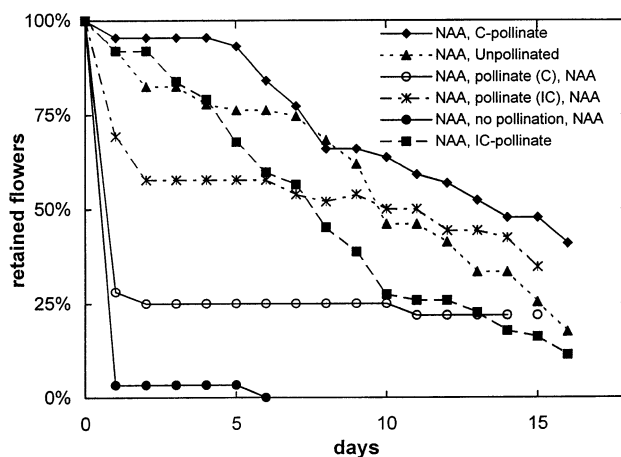


Fig. 3. The time course of 1 mM NAA application relative to the time of anthesis and pollination showed that application 1 day prior to pollination (\blacklozenge , \blacksquare) resulted in the greatest flower retention, even in unpollinated flowers (\blacktriangle). NAA application 2 days prior to pollination was less effective in compatibly pollinated (\circ) and incompatibly pollinated ($*$) flowers and led to the formation of lower plateaus of flower retention (60 and 25%, respectively). A second NAA application 3 days after anthesis (\bullet) did not prolong flower retention.

(Fig. 3). Instead of completely abscising within 3 days (Figs 1, 2), unpollinated flowers abscised gradually such that after 10 days 50% of the flowers were still intact. Both incompatibly and compatibly pollinated flowers remained intact much longer than controls. The abscission rate of compatibly pollinated flowers was lower than for incompatibly pollinated flowers.

Application of NAA 2 days prior to anthesis was not as effective in flower retention even when followed by a second application 1 day after pollination (Fig. 3). Despite the initial rapid loss of flowers, the NAA pre-treatment resulted in the retention of flowers about twice the amount of untreated controls (see Fig. 1C). Flowers that had been retained after the first 2 days persisted for almost a week before abscission resumed. The time and concentration dependence indicate that the floral physiology of *T. cacao* is very responsive to auxin. Presumably, the application of auxin alters the endogenous concentration of auxin. In order to verify that a clone's propensity to produce flowers depends on the endogenous auxin content, the endogenous auxin levels in flowers from high-flower producers such as TSH-565 were compared with low-flower producers (clones CC-10, SGU-54 and EEG-29). There was a strong negative correlation between the number of flowers produced and the endogenous IAA content (Table 1) but the ABA content did not differ between the two sets.

Table 1. Endogenous concentration of IAA and ABA in clones of *T. cacao* L. Analyzed were clones with a high (TSH-565) and low (CC10, EEG29, SGU-54) number of flowers, average of 3 determinations \pm SD with ca 20 flowers each. The differences in the IAA content were significant at $P \leq 0.05$ (*). The ratio between the number of flowers per naturally developing pod was kindly provided by A. Daymond, Department of Horticulture, University of Reading.

Clone	IAA [ng g^{-1} DW]	ABA [$\mu\text{g g}^{-1}$ DW]	Flowers per pod
TSH-565	5.7 ± 1.3^a	3.1 ± 1.6	>2 000
CC10, EEG29, SGU-54	13.7 ± 5.9	5.3 ± 0.8	48, 68, 40



Fig. 4. A flower cushion of *T. cacao* infested by *C. perniciosus* shows non-viable fruit and the outgrowth of a false branch. The flowers, fruit and branch eventually dry up but do not abscise. Bar = 3 cm.

Application of benzylaminopurine

The fungal infection of *T. cacao*, commonly known as witches' broom disease (*Crinipellis perniciosus*), causes loss of apical dominance and an increase of initial fruit set (Fig. 4). Both phenomena are typically attributed to the interaction between auxin and cytokinins. Based on these observations, we tested the effect of BAP alone and in conjunction with auxin. Despite the conjecture that cytokinins promote fruit development, spray application of BAP reduced flower retention compared with all other treatments (Fig. 5). There was no difference between the application of 10 (data not shown) and 100 μM BAP (Fig. 6). Combined application with NAA alleviated the BAP-related acceleration of abscission. Interestingly, after BAP application, a fraction of incompatibly pollinated flowers was retained for about 1 week, which constituted a lower level of abscission than with other conditions or pollination (Fig. 6).

Fruit formation

In a certain percentage of successfully pollinated flowers, *Theobroma* develops fruits despite the fact that the zygote is formed 2–3 days after pollination and the first division of the zygote does not occur until 40–50 days after pollination (Cheeseman 1927). Therefore, a signal must be present that prevents abortion and stimulates fruit development. In all cases, swelling and greening of the carpels, i.e. the first signs of fruit development were observed after 6–8 days. After an

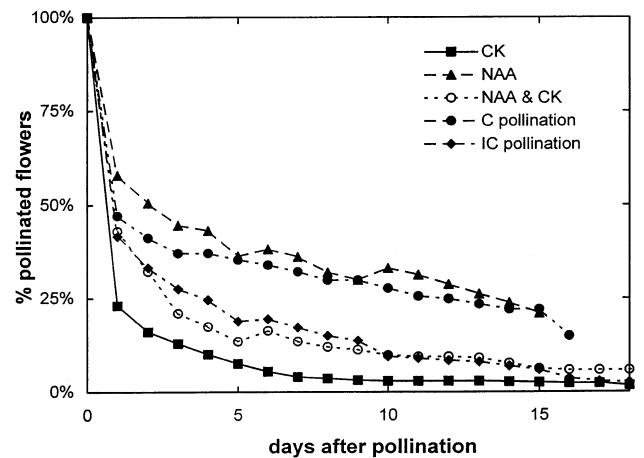


Fig. 5. The time course of abscission of flowers when BAP (CK), NAA or a combination of both was applied to compatibly pollinated flowers, compared with incompatible and compatible pollination without application of growth regulators. BAP reduced the flower retention compared with controls.

initial peak, the fruits either continued to grow or dried up and abscised in a biphasic manner (Fig. 7). As expected, compatible pollination produced the greatest number of fruits and about one-fifth of the flowers increased in volume but growth continued only in about half of the initial number. Application of NAA or incompatible pollination induced the same time course of initial fruit development. BAP either alone or in conjunction with NAA resulted in a single, more uniform distribution of fruit development. However, in all cases after 15 days the fruits that continued to develop accounted for less than 5% of the pollinated flowers.

Compared with the fraction of naturally occurring fruits, manually pollinated flowers produced 50–75 times the number of initial fruits. Selfed flowers produced about 1% of fruit, which exceeds the naturally occurring ratio between flowers and fruits after compatible pollination. However,

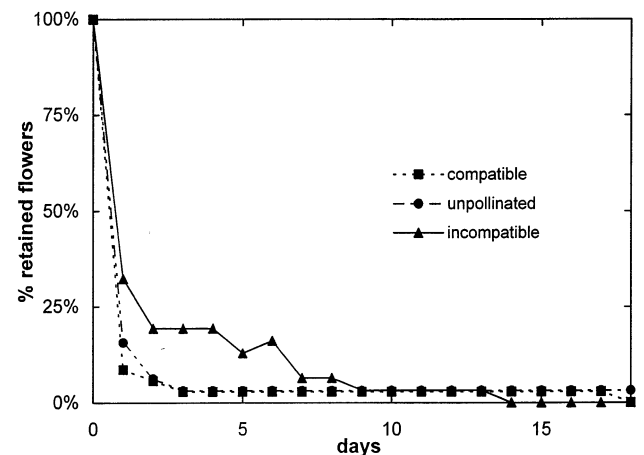


Fig. 6. The beneficial affect of cytokinin treatment in *T. cacao* was revealed only after incompatible pollination, where between 10 and 20% of flowers were retained for about 1 week. However, after 2 weeks these flowers were retained to a lesser extent than unpollinated or compatibly pollinated flowers.

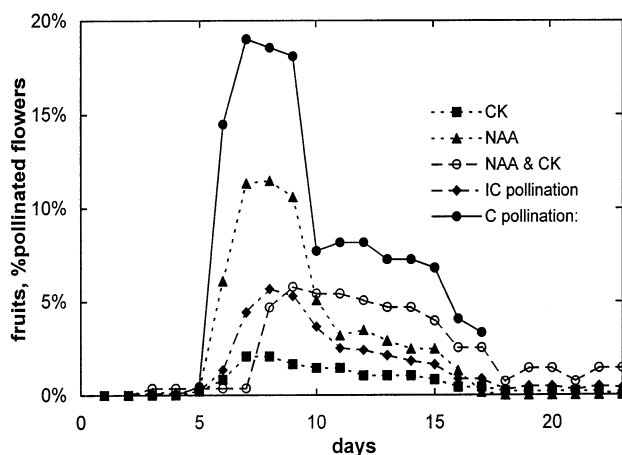


Fig. 7. Fruit development in *T. cacao* separated according to treatment, regardless of the type of pollination (■, ▲, ○) and according to pollination, regardless of treatment (◆, ●). Fruit development became first visible about 6–7 days after pollination. Incompatible pollination clearly leads to the initial stages of fruit formation in a relatively large percentage of flowers. The combination of NAA and BAP, regardless of pollination type, resulted in the largest percentage of retained flowers/fruits after >20 days.

because of the limited observation time we cannot claim that the initially present fruits indeed developed viable seeds. After 3 weeks, the largest fruits from incompatible, NAA-supported pollinations were approximately 12 mm in length. After 4 months, 6 fruits from ‘incompatible’ pollinations reached a size of 16 cm, which is average for that age.

If the flower develops into a fruit, one should be able to detect early morphological changes in the ovules. Such a change has been observed in the hypostase, a modification of the chalazal end of the ovule. In unpollinated or incompatibly pollinated flowers, the hypostase exhibits strong fluorescence (Fig. 8A), presumably because of a high content of callose and/or lignin (Tiwari 1983). In developing fruits the hypostase breaks down (Fig. 8B,C), presumably facilitating the delivery of nutrients into and export of hormonal signals out of the developing ovule. Because *Theobroma* may have a double incompatibility response at pollination and after the fusion of gametes, the hypostase could play a role in fruit development and clonal incompatibility.

Discussion

Abscission of flowers occurred within 2–3 days after anthesis and even after hand pollination, only a small share of flowers develops into a fruit. The vast number of flowers that abscise may result from a predisposition to limit fruit development. Consequently, only under the best of circumstances may the necessary physiological signals be able to override the abortion signal. This reasoning is supported by the responsiveness of *Theobroma* to applied auxin and possibly cytokinins.

Because ABA application increased flower retention for a few days (Fig. 1), both pollination and subsequent pollen tube growth may represent an additional stress signal, similar to the accelerated abortion after pollination. In unpollinated flowers, between 50 and 75% of the observed flowers remained attached after 2 days, whereas either compatible or incompatible pollination resulted in a 25–50% retention.

The application of growth regulators may not always be physiologically relevant because of the high quantities that can be provided. The application of especially NAA was within a reasonable range, because the number of retained flowers showed a strong concentration dependency, ranging from no effect (10^{-5} M) to a saturating effect at 5×10^{-3} M NAA.

The timing of NAA indicated that early application had the strongest effect, delaying flower formation and facilitating fruit development. Exogenous auxin application appears to overcome the tendency to abort flowers because retained flowers did not abscise but dried up on the maternal plant. These observations are consistent with the following scenario. The auxin content in developing buds is highest and depends on the number of flowers formed (see Table 1). As the buds mature, the auxin level declines allowing the initiation of the abscission process. At anthesis, the auxin content is low and flower retention as well as fertilization and possibly fruit development depend on an external source or signal such as suitable pollination or pollen growth. If a sufficient number and/or quality of pollen reach the ovules, fruit development is initiated. This may explain the large number of fruits that were observed even after incompatible pollination. Therefore, the quality of pollination can depend on two factors, the extent of pollen compatibility and the

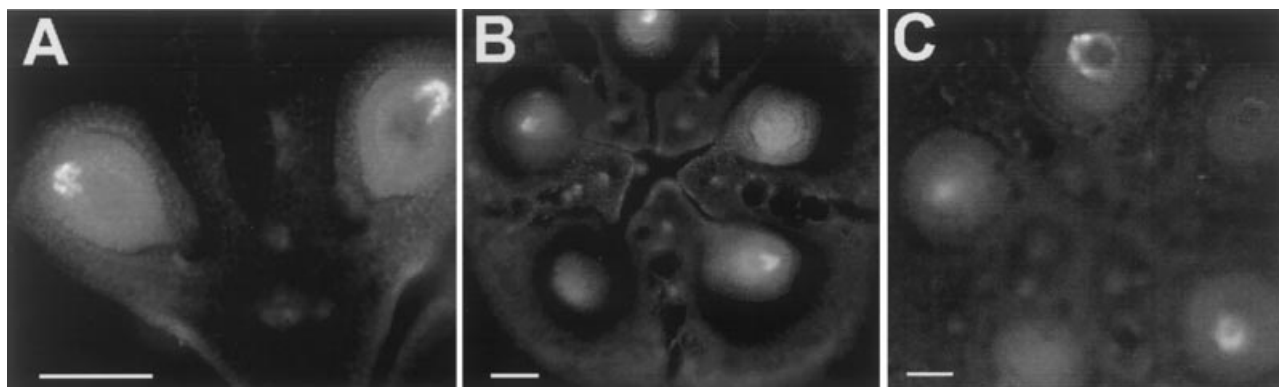


Fig. 8. (A) Epifluorescence of the hypostase 24 h after an incompatible pollination; (B) epifluorescence of the hypostase 24 h after an incompatible pollination. Note that only 3 of the 5 visible ovules show fluorescence. (C) Forty-eight hours after compatible pollination the ovules have grown and fill the locules. The fluorescence of the hypostase is markedly reduced. Bars = 25 μ m.

number of pollen grains deposited onto the stigma. The latter condition would explain the observed mentor effect, i.e. that fusion of self-gametes is possible if a sufficient number/quality of pollen is provided (Lanaud et al. 1987). As the flower ages, the decrease in auxin develops into an increasingly stronger barrier to flower retention, fertilization and fruit development. This concept may be experimentally verified by comparing pollination success and fruit set when pollination is carried out in increasingly older flowers, ranging from unopened flowers to 1 day after anthesis. Alternatively, auxin could be determined over the time of flower development.

Since flower production represents a considerable investment for the plant, the number of flowers may affect the allotment of auxin for each flower. This would explain the lower auxin content in high-flower producers and a significantly higher auxin content in low-flower bearing clones. Therefore, clones like TSH-565 that produce a large number of flowers (Table 1) can experience a twentyfold improvement in fruit development compared with the naturally occurring fruit set, despite a still low ratio between fruits and flowers, ca 1%.

A similar concept could pertain to the development of the hypostase. At low auxin levels the hypostase can form and then function as barrier. In contrast, the hypostase may not form or be less intense at high auxin levels. Auxin could also facilitate the degradation of the hypostase. This speculation should be verifiable by future investigations.

Despite the strong effect of auxin on flower viability and fruit development, the initial level of fruit set was not maintained. This decline indicates that in addition to auxin, other factors are needed to support the developing fruit. The effect of the fungal pathogen *C. perniciosa* on fruit and false development of branches (see Fig. 4) suggests the involvement of cytokinins. However, the application of BAP did not reveal any substantial effects aside from the extended fruit persistence after joint application with NAA. As there are many different agents with cytokinin activity, it is possible that either the correct cytokinin has not been identified or that the applied quantity or concentration was inadequate.

Despite the emphasis on the hormonal conditioning in the self-incompatibility system, there must be some genetic factor that determines the success of pollination. Although the result of any pollination can only be one of two outcomes, fruit set or no fruit set, the Mendelian distribution of an SI gene allows for 4 classes of pollinations, one that leads to fusion in all ovules (100%) and results in the development of fruit. All other combinations (50, 25 and 0% fusion) could result in incompatible gametes and non-fusion of egg and sperm would result in abortion. Although less than 100% fusion would typically result in flower abscission, the 50 and 25% non-fusion may be amenable to physiological modification. This physiological modification or pre-conditioning

would explain the mentor effect (Lanaud et al. 1987) and explain the strong influence of growth regulators on the flower retention and fruit set.

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References

- Aneja M, Gianfagna T, Ng E, Badilla I (1992) Carbon dioxide and temperature influence pollen germination and fruit set in cocoa. *HortScience* 27: 1038–1040
- Aneja M, Gianfagna T, Ng E, Badilla I (1994) Carbon dioxide treatment partially overcomes self-incompatibility in a cocoa genotype. *HortScience* 29: 15–17
- Baker RP, Hasenstein KH, Zavada MS (1997) Hormonal changes after compatible and incompatible pollination in *Theobroma cacao* L. *HortScience* 32: 1231–1234
- Bouharmont J (1960) Recherches cytologiques sur la fructification et l'incompatibilité chez *Theobroma cacao* L. Publications de l'Institut national pour l'étude agronomique du Congo No. 89
- Cheeseman EE (1927) Fertilization and embryogeny in *Theobroma cacao*, L. *Ann Bot* 16: 107–126
- Cope FW (1958) Incompatibility in *Theobroma cacao*. *Nature* 181: 279
- Cope FW (1959) Nuclear fusion and non-fusion in *Theobroma cacao* L. *Nature* 183: 1540
- Cope FW (1962a) The mechanism of pollen incompatibility in *Theobroma cacao* L. *Heredity* 17: 157–182
- Cope FW (1962b) The effects of incompatibility and compatibility on genotype proportions in populations of *Theobroma cacao* L. *Heredity* 17: 183–195
- deNettancourt D (1977) Incompatibility in Angiosperms. Springer-Verlag, New York, NY. ISBN 0-387-08112-7
- Dickinson HG, Lewis D (1975) Interaction between the pollen grain coating and the stigmatic surface during compatible and incompatible interspecific pollinations in *Raphanus*. *J Linn Soc* 7: 165–175
- Glendinning DR (1972) Natural pollination of cocoa. *New Phytol* 71: 719–729
- Knight R, Rogers HH (1953) Sterility in *Theobroma cacao* L. *Nature* 172: 164
- Knight R, Rogers HH (1955) Incompatibility in *Theobroma cacao* L. *Heredity* 9: 69–77
- Lanaud C, Sounigo O, Amefia YK, Paulin D, Lachenaud P, Clement D (1987) Nouvelles données sur le fonctionnement du système d'incompatibilité du cacaoyer et ses conséquences pour la sélection. *Café Cacao Thé* 31: 267–277
- Martin FW (1959) Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technol* 34: 125–128
- Opeke LK, Jacob VJ (1967) Studies on methods of overcoming self-incompatibility in *Theobroma cacao* Linn. 2e Conférence International sur les Recherches Cacaoyères, pp. 356–359
- Pound FJ (1932) Studies on the fruitfulness in cacao II. First Annual Report on Cacao Research (Trinidad), pp. 26–28
- Tiwari SC (1983) The hypostase in *Torenia fournieri* Lind: A histochemical study of the cell walls. *Ann Bot* 51: 17–26
- Warren J (1994) Isozyme variation in a number of populations of *Theobroma cacao* obtained through various sampling regimes. *Euphytica* 72: 121–126
- Warren J, Sunita MK (1995) Isozyme markers for self-incompatibility and yield in *Theobroma cacao*. *Heredity* 74: 354–356