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Carbon Dioxide Treatment Partially Overcomes Self-incompatibility in a Cacao Genotype

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Abstract. **Cacao *(Theobroma cacao* L.) contains self-compatible and self-incompatible genotypes. In the greenhouse, pollen germination and fruit set failed to occur after self-pollination of an incompatible genotype (IMC 30); however, if the self-pollinated flowers were enclosed in plastic vials for 6 h after pollination, pollen germination was 95% The promotive effect of enclosed pollination on pollen germination was due to the accumulation of CO_2 (8.9 %** v/v). **Despite the high rate of pollen germination, fruit set was only 45%. Seeds produced from self-pollinations using this technique were viable, with 95% germination. Cross-pollination with 'Amelonado' pollen resulted in 100% pollen germination and 46% fruit set. Enclosure of cross-pollinated flowers did not improve the percentage of fruit set. Sections made through the ovary 48 h after enclosed self-pollination indicated that the majority of ovules contained a zygote; however, some ovules still contained unfused male and female gametes and polar nuclei. Self-incompatibility in this genotype is expressed at two stages in the process leading to fruit set. The first is at the pollen germination stage and can be overcome by CO_2 treatment; the second is at the gametic fusion stage.**

Self-incompatibility in cacao, which was first reported by Pound (1931), has been described as late-acting (i.e., the incompatibility reaction is not expressed until gametic fusion is initiated) (Cope, 1962a, 1962b). Pollen tubes of compatible and incompatible pollinations grow equally well through the style; however, incompatible self- or incompatible cross-pollinations result in 25% to 100% nonfusion ovules-ovules that have received male nuclei but have not undergone syngamy.

Glendinning (1960) and Lanaud et al. (1987) attempted to self-pollinate self-incompatible genotypes by using mixtures of pollen from a compatible genotype and its own pollen. Using this method, some selfed seeds were obtained.To our knowledge, no other report exists about overcoming self-incompatibility in cacao, although various techniques have been used in other plant species (see reviews by de Nettancourt, 1977; Shivanna and John, 1985).

We reported that enclosing flowers after self-pollination more than doubled the percentage of fruit set in cacao (Aneja et al., 1992); an event that occurred due to the accumulation of CO_2 as a result of flower respiration. There have also been reports that incompatibility in other plants can be overcome by CO, treatment (Dhaliwal et al., 1981; Douglas and Connolly, 1989; Nakanishi and Hinata, 1973; Palloix et al., 1985; Taylor, 1982). Therefore, we have investigated the effect of CO_2 treatment, via enclosure, on pollen germination and fruit set in a self-incompatible cacao genotype.

Materials and Methods

Plants of two cacao genotypes, self-incompatible IMC 30 (obtained from the Plant Introduction Center, Miami) and self-compatible 'Amelonado', were grown in a greenhouse at a night minimum of 20C and a day maximum of 35C in 20-liter plastic containers filled with peatmoss, vermiculite, aged pine bark, and perlite (Fafard Mix no. 50; C. Fafard, Springfield, Mass.). Trees were fertilized every 2 weeks with 200 mg of 15N-6.6P–12.5 K/liter.

Intact or excised freshly opened flowers were self or cross-pollinated by hand around 9:00 AM. The staminodes were removed with jeweler's forceps to gain easy access to the pistil. For the enclosed pollinations, the pollinated flowers were covered with a 6-ml plastic vial fitted with a serum stopper, and firmly attached to the stem with modeling clay. Excised flowers were placed directly in vials following pollination. After 6 h, a l-ml gas sample was removed and analyzed for CO_2 by gas chromatography.

We observed the growth of pollen tubes in the pistil after fixing the tissue in 11% Na_2SO_3 for 24 h. We opened the styles by lengthwise dissection after washing them with water and staining them with 0.1% aniline blue in 0.03M K_3PO_4 (Bowerman, 1975). The preparations then were observed under a fluorescent microscope (Leitz, Wetzlar, Germany).

Histological studies of the ovule and embryo sac were performed on 2-µm-thick glycol methacrylate sections. Pistils harvested after 24,48, and 72 h of pollination were fixed in 10% (v/v) precooled acrolein at 0C for 24 h. Following dehydration, the material was infiltrated and embedded in glycol methacrylate (Feder and O'Brien, 1968). Sections were cut using glass knives, stained with periodic acid–Schiff's reagent (PAS), and counterstained with 1 % aniline blue (Fisher, 1968). Photomicrographs were taken on a photomicroscope (Zeiss, Germany). The fruits that had set after enclosed self- and cross-pollinations were left on the tree to ripen. The fresh weights of ripe fruits and seeds were recorded along with the percentage of seed germination and the percentage of fruit that wilted before ripening. Flowers from a group of 36 trees were used as they became available over 4 months. Treatments were randomized over time, and the data were analyzed by orthogonal partitioning of the likelihood ratio chi square (G statistic) as described by Shaffer (1973).

Results

Pollen grains from self-pollination of the self-incompatible clone did not germinate in vivo (Table 1, Fig. 1A) or after pollination of excised flowers. However, if intact or excised flowers were enclosed after pollination, pollen germination was at least 95%. (Table 1). Germination began ≈6 h after pollination (Fig. lB), and within 1 h later, pollen tubes were seen at the base of the style. The CO_2 concentration rose from ambient to 8.9% in 6 h. Placing a NaOH-saturated wick in the vials prevented pollen germination (data not shown) as previously reported for 'Amelonado' (Aneja et al., 1992).

Within 24h after enclosed self-pollinations, male gametes were released from the pollen tubes, but syngamy or triple fusion was not observed in any of the ovules (Fig. 1 C). One of the synergids degenerated by this time, being characterized by an intense staining reaction

Table 1. Effect of CO_2 treatment on pollen germination and fruit set in a self-incompatible cacao genotype.

Pollination treatment[z]		Pollen germination	Fruit set[y]	
CO_2	Method	(%)	Percent	No. flowers
–	Self	0 a	0.0 a	15
	Cross	100 b	45.6 b	18
+	Self	95 b	44.8 b	34
	Cross	100 b	38.4 b	26

[z]+ CO_2 treatment given by enclosing the flowers in plastic vials on the tree. 'Amelonado' was used in the cross pollinations.
[y]Percentage of flowers in which fruit set occurred. Mean separation by orthogonal partitioning of the likelihood ratio chi-square.

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for proteins. Fusion of male and female gametes occurred 48 h after pollination (Fig. lD), and the open self-pollinated flowers from the self-incompatible clone had abscised. The zygote was binucleolate, and the staining reaction of the cytoplasm showed an increase for total proteins. The majority of the ovules contained a zygote 48 or 72 h after pollination; however, some ovules in which male and female gametes were still unfused were encountered with the free nuclei lying in close proximity to each other in the embryo sac (Fig. 1 E and F).

Despite 95% pollen germination for enclosed self-pollinations, the percent fruit set was <50% (Table 1). Increasing the level of CO_2 inside the vial (by injecting 1 ml of 15% CO_2) or increasing relative humidity (by adding a water-saturated wick) did not increase pollen germination or the percent fruit set (data not shown). Enclosing the flowers for an additional 6 h also did not increase fruit set; in fact, this treatment prevented fruit set entirely (data not shown).

When the flowers of the self-incompatible clone were cross-pollinated with 'Amelonado' pollen, percent fruit set was not significantly different from that obtained by enclosed self-pollination (Table 1). Moreover, enclosing the cross-pollinated flowers did not further enhance fruit set, as it did when 'Amelonado' flowers were self-pollinated (Aneja et al., 1992).

The seeds from the selfed incompatible clone obtained by the enclosed pollination technique had a high rate of viability, and 95% germinated. Although the fresh weight of the fruit obtained by enclosed self-pollination of the self-incompatible genotype was significantly greater than that of 'Amelonado' fruit from self-pollination (523 vs. 323 g, n = 35), the fresh (≈2.0 g) and dry (≈ 1.34 g) weights of the seeds, the number of seeds per fruit (33.7 vs. 32.6), and the percentage of fruits that wilted before maturity (40.6 vs. 46.5) were not significantly different.

Discussion

Enclosure of self-pollinated cacao flowers for 6 h after pollination partially overcomes the self-incompatibility reaction and allows the production of selfed seeds. Carbon dioxide is the likely agent responsible for this effect, as CO_2 accumulates in the vial to at least 8% in 6 h and absorbing the CO_2 as it is produced prevents pollen germination and fruit set (Aneja et al., 1992). A high level of CO_2 applied for a short period after pollination also has been shown to overcome sporophytic self-incompatibility in *Brassica oleracea* L. Capitata Group (Nakanishi and Hinata, 1973; Palloix et al., 1985; Taylor, 1982) and *B. campestris* L. (Dhaliwal et al., 1981), and gametophytic self-incompatibility in *Trifolium repens* L. (Douglas and Connolly, 1989). Ethylene has been reported to overcome gametophytic self-incompatibility in *Lycopersicon peruvianum* (L.) Mill. (Webb and Williams, 1988; Williams and Webb, 1987); however, we were not able to detect the presence of ethylene during the 6-h enclosed pollination period in cacao flowers (Aneja et al., 1992) and concluded that it did not play a significant role in cacao pollen germination.

In this study, we did not find any germinated pollen on flowers left open on the tree after self-pollination of the self-incompatible clone. However, Cope (1962a, 1962b) reported similar rates of pollen germination and tube growth in incompatible and compatible pollinations. We have no explanation for this difference. For events after pollen germination, our results were similar to Cope's results (1962a, 1962b), particularly the presence of some ovules with unfused nuclei inside the embryo sac 72 h after pollination.

From this study, it seems that self-incompatibility occurs at two stages in the process leading to fruit set: first at the stage of pollen germination, which can be overcome by CO_2, and second at the stage of gametic fusion as has been previously reported (Cope, 1962a, 1962b). This hypothesis explains the low rate of fruit set (45%) from enclosed self-pollinations of the self-incompatible genotype—a partial block to fruit set still exists at the stage of gametic fusion. Similarly, enclosed cross-pollinations using 'Amelonado' pollen are no more effective than open cross-pollinations. Although pollen from this source germinates on the stigma of the self-incompatible clone without CO_2 treatment, 'Amelonado' exhibits a certain level of cross-incompatibility, perhaps at the stage of gametic fusion.

The technique of enclosed pollination of cacao flowers needs to be tested under field conditions. In *Brassica*, CO_2-generating bombs have been used to overcome self-incompatibility (Nakanishi and Hinata, 1973, 1975; Nakanishi et al., 1969). Other techniques to overcome incompatibility, such as physical damage to the stigmatic surface (Roggen and Van Dijk, 1972), electric current (Roggen et al., 1972), and heat (Roggen and Van Dijk, 1976), are time consuming and require considerable skill. The method presented in this paper is simple, rapid, and effective in producing selfed seeds from a self-incompatible genotype under our greenhouse conditions, and if of general use, it may be useful in cacao breeding programs.

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