Theor Appl Genet (1985) 70:628–633

Effect of carbon dioxide and relative humidity on self incompatibility in cauliflower, *Brassica oleracea*

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Received January 14, 1985; Accepted February 15, 1985
Communicated by H. F. Linskens

Summary. The events of the progamic phase of fertilization have been monitored by in vitro experiments in self compatible (SC), partial self-incompatible (PSI) and self incompatible (SI) lines. The duration of the progamic phase is about 30 h. Treatment with low concentrations of CO_2 (3 to 5%) at high relative humidity (rH, 100%) had the following effects: pollen quality, which declines normally during flower ageing, was prematurely reduced; pollen adhesion and germination, both low in SI matings, were increased; the stigma callose response in SI matings was reduced to the low level of SC matings; and the number of pollen tubes in the style after SI matings significantly increased. CO_2 concentrations of 4 to 6% applied for 8, 16 or 24 h at 100% rH proved to be the most effective treatment for blocking the SI response in cauliflower.

Key words: Carbon dioxide – Relative humidity – *Brassica oleracea* – Pollen-pistil interactions – Self compatibility

Introduction

In *Brassica,* sporophytic self incompatibility (SI), in which self pollen or pollen tubes are inhibited on the stigma surface, is a useful tool for the production of commercial F1 hybrid seed. However, the existence of SI constitutes a problem in obtaining parental inbred lines, as selfing is normally possible only by bud pollination, a time-consuming and labour intensive process. A more practical method has been introduced, involving treatment of selfed mature flowers with low concentrations of CO_2, i.e. 3–5% (V/V) for 6 h (Nakanishi and Hinata 1973, 1975).

Since then, other groups have reported the enhancement of self seed set by high relative humidity, applied alone (Carter and McNeilly 1975), or in combination with CO_2 (Dhaliwal et al. 1981; Sharma et al. 1981).

The effect of CO_2 on the initial events of pollen-pistil interactions has recently been investigated by O'Neill et al. (1984). These authors found that the stigma callose response in *B. campestris,* which develops within a few hours of self pollination (Heslop-Harrison et al. 1975), is blocked by exposure to low levels of CO_2.

The present experiments are part of a research programme to clarify the effects of CO_2 on pollen-pistil interactions, in terms of pollen quality, pistil and ovule receptivity, the three features of the reproductive system that control seed production (Dumas et al. 1984). Our experiments utilized an in vitro system of pollination to explore:

- the timetable of fertilization;
- quantitative changes in key processes of the programic phase of fertilization;
- the effect of CO_2 on the stigma read-out of pollen information.

Materials and methods

Ten lines of autumn cauliflower, *Brassica oleracea* L. var. 'Botrytis', originating from five to eight generations of selfing, were grown at INRA, Rennes. Following a diallel of crosses to establish the strength of self-incompatibility (SI), ten lines showing different levels of SI were selected (Table 1). Controlled pollinations were carried out in a greenhouse, using flowers at anthesis, or 3–5 days prior to anthesis in the case of bud pollination. All pollinations were made by the direct application of dehiscing anthers to the stigma surface.

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Table 1. Lines of autumn cauliflower, *Brassica oleracea* var. 'Botrytis' tested for self incompatibility; SI, self-incompatible; PSI, partial self-incompatible; SC, self compatible

Code	Line	Breeding behaviour
1	7-339-18-5-19	SI
2	7-938-23	SI
3	8-1272-3-1	SI
4	8-1271-11-15	PSI
5	7-512-4-2	PSI
6	7-768-3-10	PSI
7	D-43-6-39-61-11-90-7	PSI
8	7-719-35-2-1-3	SC
9	7-925-5	SC
10	7-915-18-8	SC

In vitro bioassay of self incompatibility

Flowers were excised and placed on agar medium (1% agar, 100 ppm boric acid) in a glass container sealed with parafilm. A measured amount of CO_2 was injected to obtain the desired concentration. Two environments were provided: 100% relative humidity (rH) and a dry atmosphere over silica gel. Relative humidity was monitored with a thermohygrometer. Glasshouse air was employed as a control atmosphere, rH 40 to 60%, at 22–25 °C.

Assessment of pollen quality

The fluorochromatic reaction (FCR) test was employed using pollen dispersed in 10% sucrose as described by Heslop-Harrison et al. (1984).

Cytological monitoring of pollen germination and tube growth

Pistils were fixed at various times after treatment in ethanol: acetic acid (3 : 1) for 30 min, then washed in 70% ethanol and cleared in 10 N NaOH for 1 h at 60 °C (Martin 1959) and processed for decolorized aniline blue fluorescence (ABF) as described by Linskens and Esser (1957) (see review by Dumas and Knox 1983). The following events were monitored:

Adhesion: the number of pollen grains adhering to the stigma was assessed after processing by the ABF method, either at 2 h or 24 h after pollination.

Germination: The percentage germination was estimated from a sample of 25 pollen grains scored per stigma. Pollen was considered to have germinated when tube length equalled or exceeded grain diameter.

Pollen tube penetration of the stigma: this parameter was estimated by direct counting of tube numbers in a transect towards the base of the style after processing by the ABF method.

Callose response: callose formation was scored by recording two different parameters: (a) the number of stigma papillae showing specific fluorescence: 0, none; 1, 1 to 10 papillae; 2, more than 10 papillae; 3, whole stigmatic surface; (b) an estimate of the fluorescence intensity of callose lenticules: 0, weak and diffuse; 1, faint but detectable; 2, moderate; 3, intense fluorescence.

Statistical analyses were performed on data obtained from these experiments. Batches of five pistils were employed for each replicate of treatments and for each different genotype used. Four different replicates were made for each treatment. Duncan's (1955) multiple range test and a two-way analysis of variance were employed to determine the significance of results the results. Means were transformed to Ln (x + 1) where their variances were not homogeneous.

Results

Timetable of fertilization

Pollen germination. After a self compatible (SC) mating (Fig. 1 a) maximal germination, i.e. approximately 80%, was reached by 6 h. After a compatible cross, similar germination percentages were obtained (Fig. 1 b). Following selfing in a high SI line, germination reached 22% by 6 h, and rose slowly to >40% by 24 h (Fig. 1 c). Results for self pollination in a partial SI line were similar (Fig. 1 d).

Pollen tube growth through the style. In SC or cross compatible matings, the number of pollen tubes observed in style transects reached a maximum by 24 h after pollination, when up to 50 tubes were observed (Fig. 1 a, b). In contrast, following selfing of a high SI line, no tubes penetrated into the style, and in a partial SI line, fewer than 10 tubes were present.

The earliest record of fertilization, detected by pollen tube entry into the ovules, occurred at 30 h after pollination. Pollen tubes most frequently entered ovules at the base of the ovary first, possibly indicating that maturation of the ovules occurs initially in basal ovules. Thus, in breeding experiments, a convenient time to test cytologically for SI is at 24 h after pollination under the conditions used here.

Effect of CO_2 on pollen quality

A decline in FCR scores from greater than 80% to less than 20% (Fig. 2 a) indicates a progressive loss of pollen quality from the time of petal opening until flower withering, about 90 h later. During the first 24 h, there was only a slight loss of quality, to approximately 70%, showing that this represents the best time period for pollen to be employed in breeding experiments.

In the presence of 6% CO_2 and 100% rH, there was an accelerated loss of pollen quality to less than 50% after 24 h and less than 30% after 48 h (Fig. 2 b). However, pollen from newly opened flowers when tested after 24 h exposure to CO_2 followed by 24 h in air, showed almost as high a quality as pollen from untreated plants. This suggests that the period in air provides a sufficient recovery period for normal pollen quality to be restored in developing buds. There could be two alternative explanations of this observation: 1) pre-anthesis pollen stage is not CO_2 sensitive; 2) CO_2 is not penetrating closed buds.

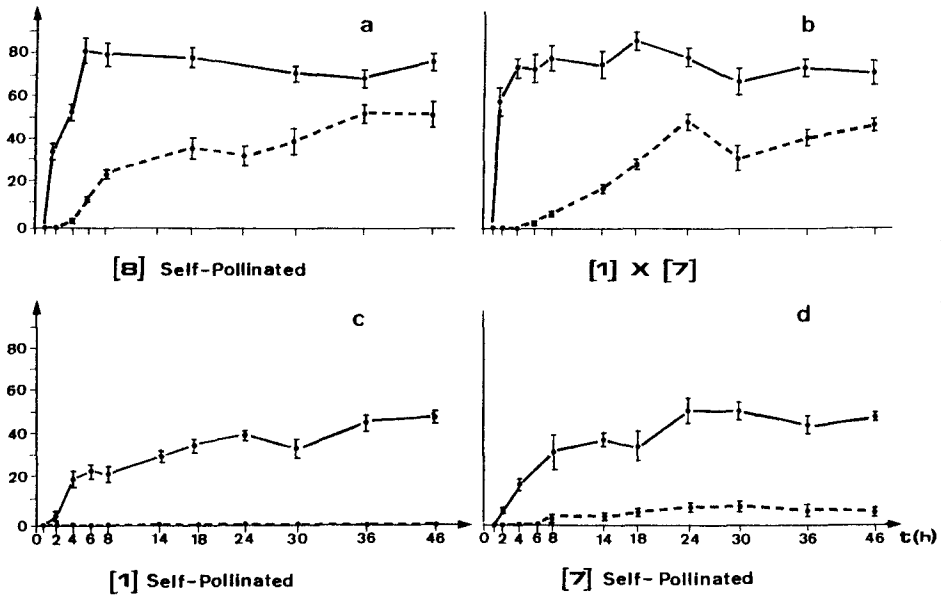

Fig. 1 a–d. Time course of pollen germination and pollen tube growth in style following various types of pollination in *Brassica oleracea* var. 'Botrytis': **a** SC line selfed; **b** compatible cross between SI and PSI lines; **c** SI line selfed; and **d** PSI line selfed. Percent germination is indicated by *solid line;* pollen tube score in style transect by *broken line.* Each point is the mean (±SE) of 4 replicate treatments observed by fluorescence microscopy

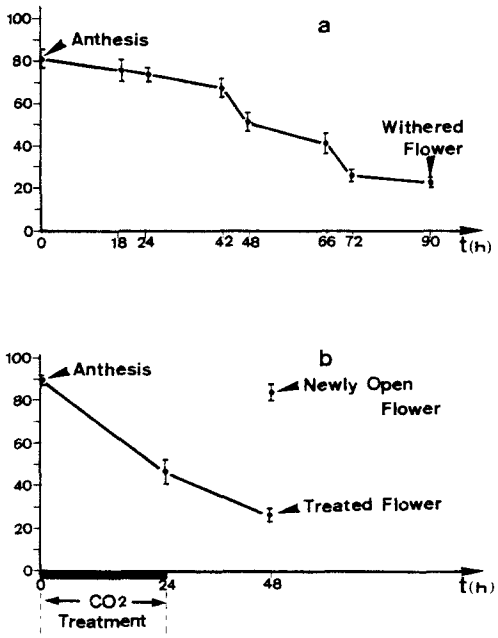

Fig. 2. Estimates of pollen quality of *B. oleracea* var. 'Botrytis' **a** during flower ageing in air; **b** in 6% CO_2 and 100% rH for 24 h. Each point is the mean of 10 lines tested, ± SE

Effects of CO_2 and rH on fertilization

Pollen adhesion to the stigma, tested at 2 h after pollination, when tube penetration had not occurred, was high in SC matings, and low in SI matings (Table 2). Following CO_2 application, self pollen adhesion was promoted (Fig. 3 a). Adhesion of self pollen increased significantly in concentrations of CO_2 up to 2%, and remained high even at 15% CO_2. However, at 20% CO_2, adhesion was lower than that found in air.

The stigma callose response, high following SI matings, was reduced to the low level of SC matings when CO_2 concentration was increased to 4 or 6% (Table 2). Different levels of CO_2 had a significant effect on stigma callose score ($F=35.5$, $F.05=2.19$), as had duration of application ($F=15.9$, $F.05=2.7$). The response was halved in 2% CO_2 (Fig. 3 b). Pollen tube fluorescence was also weak in the presence of 4% CO_2; callose plugs were smaller and there was no callose fluorescence along the walls of the tubes, making tube counting difficult.

At low concentrations of CO_2 (2 to 6%) self pollen germination and tube growth in the style were promoted (Fig. 3 c, d; Table 2). By 24 h, pollen germination scores for compatible pollen were high, greater than 85%, but less than one third of the level for self pollen (Table 2). In the presence of 6% CO_2, germination was stimulated to nearly 50% and CO_2 concentration effects on germination were found to be significant at the 1% level ($F=16.7$, $F.05=2.19$). The duration of CO_2 application was also shown to affect pollen germination ($F=4.7$, $F.05=2.7$). CO_2 concentration had a significant effect on the number of pollen tubes in the style ($F=6.52$, $F.05=2.19$). The duration of CO_2 treatments in relation to style penetration was also significant ($F=10.3$, $F.05=2.7$). The optimum treatment for overcoming the SI response was found to be 4 to 6% CO_2 applied for 8, 16 or 24 h at 100% rH. However, the number of pollen tubes in self styles following optimum CO_2 treatment was still only approximately 15% of that observed in a compatible cross (Table 2). The increased number of

Table 2. Effect of CO_2 and rH on the events of the programic phase of fertilization in *Brassica oleracea* var. 'Botrytis'. Duncan's multiple range test of the stigma data. (a, b, c, d, e): values with different letters as postscript are significantly different ($P<0.05$)

Fertilization event	Self pollination				Cross pollination (air)	Unpollinated control
	Air	100% rH	6% CO_2 (dry)	6% CO_2 + 100% rH		
Adhesion[a]	11.8 a	21.2 a (6.6) a	105.0 b	94.2 b (23.0) b	175.0 c (47.8) c	0
% Germination	34 a	45 a	48 a	55 b	85 c	0
Callose response	5.4 a	4.4 b	3.2 c	1.4 d	1.4 d	0.4 e
No. of tubes in style	0.2 a	0.2 a	2.2 b	9.2 c	58 d	0

[a] Estimated at 24 h, values at 2 h given in parentheses

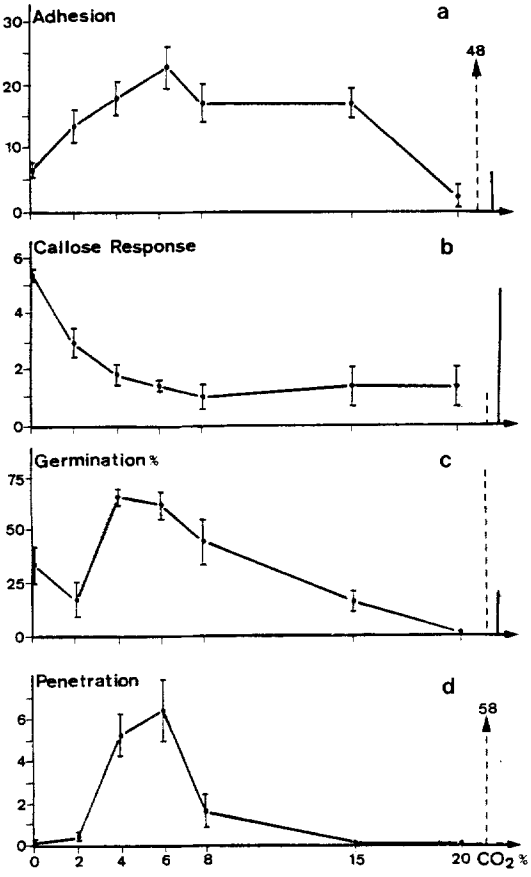

Fig. 3a–d. Effect of increasing concentrations of carbon dioxide on the events of fertilization in *B. oleracea* var. 'Botrytis'. Data were obtained from self-pollination of SI lines; **a** pollen adhesion at 2 h after pollination; **b** stigma callose response at 8 h after pollination; **c** pollen germination at 24 h after pollination; **d** self pollen tube penetration and growth in style at 24 h after pollination. Abscissa values are arbitrary scores described in text. Each point is the mean of 5 stigmas (± SE). *Vertical lines* at RHS of graphs show control means for SC self pollen (*broken line*) and SI self pollen (*solid line*) when maintained in air

Table 3a, b. Analysis of pollen tube growth in style after various carbon dioxide treatments. **a** Duncan's multiple range test of treatment means. For analysis, values were transformed to Ln (x + 1), but original data are given. Values with different letter as postscript (a, b, c) are significantly different ($P<0.05$). **b** Analysis of variance. Data are transformed to Ln (x + 1). S = significant; N.S. = not significant

a Line	Treatment[a]						
	1	2	3	4	5	6	7
6 (PSI)	0.4 a	1.4 a	0.0 a	0.0 a	1.0 a	0.0 a	14.0 b
7 (PSI)	0.0 a	8.4 b	5.6 b	7.6 b	4.6 b	7.2 b	10.0 b
2 (SI)	0.8 a	0.0 a	2.8 a	2.0 a	2.2 a	0.2 a	5.8 b

b Source	S.S.	D.F.	M.S	V.R.	$P<5\%$	$P<0.1\%$
Genotype	22.87	2	11.44	13.79	S.	S.
Treatments	20.38	6	3.39	4.09	S.	N.S.
Interaction	20.69	12	1.72	2.08	S.	N.S.
Error	69.63	84	0.83			
Total	122.63	104	1.28			

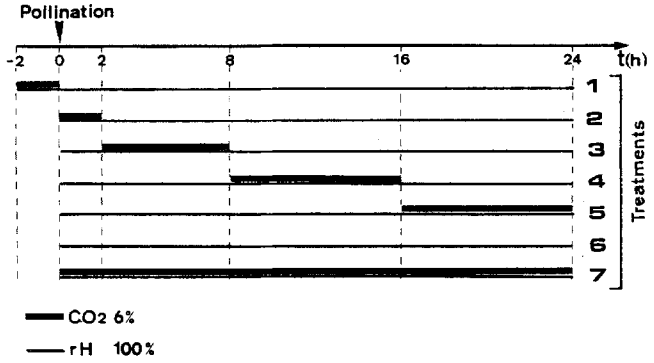

Fig. 4. Experimental design of treatments to show effects of CO_2 and rH on fertilization. Results of treatments 1–7 are given in Table 3

self pollen tubes in the style was stimulated by a narrow range of CO_2 concentrations, 4 to 6%, (Fig. 3 d).

Timing of CO_2 effects during fertilization

Brief durations of CO_2 treatment did not have as marked effects as prolonged treatment, whether applied before or during the fertilization process (Fig. 4 and Table 3 a, b). The number of pollen tubes in the style were not increased significantly by 8 h treatment when compared with air controls (Table 3 a), and tube number did not reach the same levels as pollen tubes in flowers under continuous CO_2 treatment for 24 h. The same conclusions were reached for all three SI lines available (Table 3 b).

The effect of rH in overcoming incompatibility was most noticeable in the longer CO_2 treatments-over 16 or 24 h. High rH treatment alone did not overcome the SI response (line 2) by any of the available criteria (Table 3 a) although the effects on the two PSI lines were variable.

Discussion

CO_2 blocks SI response

Low concentrations of CO_2 applied during the progamic phase overcomes the SI mechanism in cauliflower. This was first demonstrated in cabbage by Nakanishi and Hinata (1973, 1975). In cauliflower, the most effective concentrations (4–6%) are similar to those reported for *B. napus* (Dhaliwal et al. 1979) and *B. campestris* (Dhaliwal et al. 1981; O'Neill et al. 1984). Prolonged periods of CO_2 application, i.e. 8, 16 or 24 h, are most effective in blocking the SI response in cauliflower. The pollen of cauliflower showed a strong sensitivity to the low levels of CO_2 that are used to block SI. This observation is in contrast to those of Sharma et al. (1981) who found that *Amaryllis* pollen tube growth was promoted by low levels of CO_2 (< 3%).

In this study, we have examined the effects of CO_2 on the principal events of fertilization: pollen adhesion, hydration and pollen tube penetration and growth. Adhesion of self pollen of cauliflower to the stigmas is markedly enhanced, almost to SC levels, by exposure to a range of CO_2 concentrations (2–8%) although higher concentrations apparently had a toxic effect. Similar results were obtained for self pollen tube penetration and growth in the style.

The timing of CO_2 treatment is important. Treatments in which CO_2 was applied to stigmas before pollination showed no effect on the SI response, supporting the experimental findings of Ito (1981) with radish and O'Neill et al. (1984) with *B. campestris.* Recent ultrastructural observations of the pollen-stigma interface in *B. oleracea* indicate that there is a modification of the stigma surface pellicle soon after pollination (Gaude 1982; Dumas et al. 1984). This may result in water uptake by the pollen grain in contact with the pellicle, as originally proposed by Mattsson et al. (1974). Increased CO_2 levels may modify this process in self pollinations. The synergistic effect of increased relative humidity is probably due to the promotion of pollen hydration and germination, thus enhancing the effect of CO_2 in overcoming the SI response.

We can conclude that:

– CO_2 acts at the pollen-stigma interface rather than on the pollen or stigma separately;
– CO_2 treatment increases self pollen adhesion, germination and tube penetration.

CO_2 and the callose response

The stigma callose response to self pollen in cauliflower is blocked by CO_2, as shown by semi-quantitative assays. Concentrations as low as 2% strongly inhibited callose accumulation in the stigma papillae, the levels of fluorescence being comparable with those of SC interactions. Such an effect of CO_2 on the stigma callose response to self pollen has also recently been reported by O'Neill et al. (1984) in *B. campestris,* who detected differences in the stigma callose response in experiments in which CO_2 levels were monitored continuously.

Inhibition of the callose response may be brought about in two ways. First, CO_2 may modify stigma metabolism after pollen recognition, so that callose formation is prevented. Support for such an alteration in carbohydrate metabolism comes from Sharma et al. (1981) who demonstrated an increased CO_2 fixation by PEP carboxylase in *Amaryllis* pollen exposed to low levels of CO_2; and from the present observations of reduced fluorescence of both stigma papillae and self pollen tubes following CO_2 treatment.

Second, CO_2 could directly affect the interaction between stigma and pollen S-gene products, so that the signal to initiate the callose response is modified or not given. The stigma callose response in *B. oleracea* is induced specifically by self pollen or its surface component (Kerhoas et al. 1983). The increase in self pollen adhesion in the presence of low levels of CO_2 now demonstrated lends further support to this hypothesis. CO_2 may modify the S-specific stigma glycoproteins, their complementary pollen receptors, or their binding interactions (review by Dumas and Gaude 1983). CO_2 is known to affect the fluidity of plant cell membranes (Doree et al. 1972) and may have a synergistic effect with calcium ions to modify calcium transport (Mitz 1979). In animal systems, CO_2 is known to alter the structure of the egg cell membrane and thus affect its penetration by sperm cells, so modifying fertilization (Salisburg and Van Damark 1957). CO_2 has been shown to play an active role in many biological systems and has provided a new method for control of reproduction in crop plants.

Acknowledgements. We thank INRA-CNRS and the Australian Department of Education (Special Research Centres Programme) for financial support; Dr. Elizabeth Williams, Patricia O'Neill and Josephine Kenrick for valued discussion and Debbie Irvine for skilled typing.

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