Séance du 4 février 1993

PLASMA MEMBRANE THICKNESS AND FLOWERING INDUCTION IN *CHENOPODIUM RUBRUM* AND IN *SPINACIA OLERACEA*

PAR

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ABSTRACT

The leaves plasma membranes could be implicated in flowering induction process. Comparative studies have been carried out with a short day plant, *Chenopodium rubrum*, and a long day plant, *Spinacia oleracea*. Electron microscopic observations have shown modifications of membrane thickness and structure. A modification of plasmalemma sterols content also appeared during floral transition. Implications of these phenomena on plant physiology were discussed.

Key-words: *Chenopodium rubrum*, Flowering induction, Plasmalemma, *Spinacia oleracea*, Sterols.

RÉSUMÉ

Le plasmalemme foliaire pourrait être impliqué dans le mécanisme de l'induction florale. Des études comparatives ont été effectuées à la fois sur une plante de jour court, *Chenopodium rubrum*, et sur une plante de jour long *Spinacia oleracea*. Les observations faites en microscopie électronique montrent des changements de l'épaisseur et de la structure membranaire. L'analyse chimique fait apparaître une modification de la composition en stérol lors de la transition florale. Les implications de ces phénomènes sur la physiologie de la plante sont discutées.

INTRODUCTION

The flowering process depends on internal factors (i.e. genotype, hormones, nutrients) and in many plants is clearly controlled by external parameters (light-dark cycle; temperature). In spite of various studies (for review *see* BERNIER G. 1988, BODSON M. & BERNIER G. 1985), it remains largely unknown. If the transmission of signals from the leaves to the shoot apex is well established (BODSON M. & BERNIER G. 1985), the nature of a specific signal is still unknown. The presence of a florigen (hormone concept) or combinations of stimulators and inhibitors have been postulated (CHAILAKHYAN M.K. *et al.* 1985). Until now, attempts to extract any specific chemical compound remain a blow. Another theory, supported by some experiments (SACHS R.M.

1977, DEGLI AGOSTI R. *et al.* 1987), is the nutrient diversion hypothesis (BODSON M. & BERNIER G. 1985). If the free sugars usually rise up at the time of flowering induction and if this quantitative effect can induce evocation of apical meristem, it must act on specific structure. No relation of this type has been clearly demonstrated until now. Recently, some oligosaccharides which mimic hormonal effects have been extracted (TRAN THAN VAN K. *et al.* 1985). Unfortunately, a clear relation with flowering is missing. All these theories don't allow to explain the phenomenon at cellular and molecular levels.

Our laboratory has developped another approach (GREPPIN H. *et al.* 1978). The main goal is to find, under different environmental conditions, early markers (structural, biochemical and biophysical) of floral induction and expression. This approach has shown that plasma membrane could be very quickly involved in the flowering process (GREPPIN H. *et al.* 1990). For this reason, systematic studies have been undertaken on this membrane.

Experiments runned with spinach, a long day plant, have shown that structural modifications of plasma membrane, principally thickening, appear at the time of the transition from vegetative to floral state (AUDERSET G. *et al.* 1986). This thickening has been also obtained in short day by a gibberellic acid treatment (CRESPI P. *et al.* 1989), a substance known to favor evocation in spinach (METZGER J.D. *et al.* 1985). This plasma membrane modification has been observed in both the leaves and in the apical meristem (CREVECOEUR M. *et al.* 1992). In the same time, we only have found a modification of sterol content of this membrane (CRESPI P. *et al.* 1989, CRESPI P. *et al.* 1993). Some experiments have been runned to correlate these two results. We have treated plants with paclobutrazol, a substance known to inhibit or to interfere with flowering induction and sterols (HAUGHAN P.A. *et al.* 1989).

Some experiments have also been carried out for comparing with a short day plant, *Chenopodium rubrum*. We have been able to observe some modifications of plasma membrane structure and composition in response to the photoperiodic induction of flowering.

MATERIAL AND METHODS

Plant material

Spinach plants (*Spinacia oleracea* L. cv. Nobel) were grown for 4 weeks in a growth chamber with short day illumination (fluorescent tubes, Sylvania: 400 μmol. m^{-2}.s^{-1}) of 8 hrs daily. The temperature was set at 23°C. The relative humidity was maintained at ca. 80%. Plants were photoinduced by a continuous light period of 24 hrs. Control plants remained under short day condition.

Chenopodium plants (*Chenopodium rubrum*), were grown under long day condition for 3 weeks with the same illumination than spinach. Plants were photoinduced by a dark period of 12 hrs.

To dissociate the effect of light quantity from specific events of flowering process, we have mimicked flowering induction of spinach by a gibberellic acid treatment of plants (AG$_3$ 10^{-3} M, three consecutive days, two times per day). Spinach plants were

also sprayed two times with paclobutrazol (50 mg/l, *e.g.* 2 hrs before the start of prolongation of light period and after 4 hrs after this start).

For chenopodium, analysis of plasma membrane have also been runned after a red break (30 min.) given 8 hrs. after the start of the inductive dark period. This treatment is known to inhibit floral induction (CUMMING B.G. *et al.* 1965).

Plasma membrane isolation

Pure plasmalemma was prepared from a total membrane extract by separation in a two-phase system according to KJELLBOM P. and LARSSON C. (1984). This procedure was used without modification for spinach extract and with only few small changes for chenopodium (ie. 5.7% Polyethyleneglycol and 5.7% Dextran).

Electron microscopy

Aliquots of membrane fractions were prepared for electron microscopy and measurements of plasma membrane thickness were done on negatives of plasma membrane portions digitalized with an image analysis system (Biocom, Les Ullis France) as previously described by CREVECOEUR M. *et al.* (1992). A minimum of 250 measurements were averaged for each determination.

Lipids extraction and sterols analysis

Lipids extraction was made by phase separation as described by FOLCH J. *et al.* (1957) and sterols were separated and analysed by reverse phase high performance liquid chromatography essentially according to BULDER H.A. M. *et al.* (1984).

Statistics

The data were summed for each experimental series and the average values were statistically analysed by the method of variance analysis and by the Student t-test.

RESULTS

A modification of plasma membrane thickness and structure of spinach leaves (long day plant) has been clearly demonstrated. These results, shown in table n° 1 and figure n° 1A, were obtained by analysis of plasmalemma extracted from whole leaves tissues. The increase of plasmalemma thickness observed after photoperiodic induction was found to be 15% to 20% greater than control. Gibberellic acid, under non inductive short day, induces the same effect (table n° 1 and figure n° 1B). The same type of experiments has been made with *Chenopodium rubrum*, a short day plant (table n° 1 and figure n° 2). Only a very small increase of plasmalemma thickness appeared during the first dark inductive period (12 hrs dark). This small increase was not completely

abolished by a red break (table n° 1, experiment n° 3). If we use statistical test (Student-t test), the values of plasma membrane thickness were different from control values only at P≤0,025 against P≤0.005 for spinach. The thickening of plasma membrane observed in both plants was also illustrated by densitometric profiles (figure n° 3). A detailed examination of plasmalemma aspect on electron microscopic negatives give some characteristic differences in spinach but also in chenopodium after the transition to flowering. Membranes extracted from induced plants were more grumous or ruffle as shown in figure n° 3. These observations were based on image analysis after digitalization of negatives pictures. Unfortunately, it is not possible actually to quantify simply this type of information (pattern analysis).

Previous experiments have shown a modification of the sterol content in spinach plasma membrane. A decrease, during the first hours of additionnal inductive light, was followed more or less rapidly by an increase of the leaves sterol content. We only have obtained a decrease of this parameter after a three days gibberellic acid treatment of plants in short day (CRESPI P. *et al.* 1989). Previous experiments on chenopodium leaves have shown a distinct increase of plasmalemma sterol content during the first hours of floral induction by darkness (figure n° 4). No qualitative modifications of composition have been observed.

We have tempted to correlate these two events: i.e. thickening and change in sterol content in spinach. For this purpose, we have used inhibitors of sterol and/or gibberellin metabolism. Plasmalemma thickness has been examined after a treatment with two different enantiomers of paclobutrazol. The enantiomer 2S3S of paclobutrazol has no effect on the thickening observed after photoinduction. We don't find differences between plasma membrane thickness of control and light induced plants after a treatment with paclobutrazol 2R3R. This chemical provokes a significant increase of thickness in control plants and a decrease in induced ones (table n° 2 and figure n° 5).

DISCUSSION

The aim of this work was to determine if the response of a short day plant *Chenopodium rubrum* and a long day plant *Spinacia oleracea* to photoperiodic induction presents some similarities at the plasma membrane level. Electron microscopic analysis have shown that thickness and structure of this membrane are modified during floral induction by light or chemicals. These observations have been substanciated by infra-red spectrum analysis of spinach plasma membrane (BELLAMINE J. *et al.* 1993). However, if the observed effects were clear in long day plants spinach, it was less evident, but present, in short day plants chenopodium. Probably, the induction by light has a more marked impact than the induction by darkness.

What is the basis of the membrane modifications? Two aspects have to be considered: the physico-chemical and the compositionnal aspects. Membrane lipids could be found in different states: liquid-cristalline or gel-sol which are lamellar structure

and also non lamellar hexagonal H_I or H_{II} structure (LESHEM Y.Y. 1991). Analysis of these parameters have been until now carried out principally on model system, liposomes (SCHULER I. *et al.* 1991). Cellular membranes are complex mixtures of lipids, proteins, sterols, sugars and ions. They are highly structured but could certainly evolved from one homeostatic form to another ones. If this explanation could be expected, precise examination of their physico-chemical state should be runned, especially if hexagonal H_{II} structure exists in natural membranes (GORDON-KAMM W. J. *et al.* 1984).

Compositionnal analysis have shown only distinct differences in sterol plasma membrane content in relation to floral induction. However if quantitative variations in lipid saturation level or protein content could not be removed the question rises to know whether plasmalemma thickness and sterol content were correlated. Many experiments, runned in parallel, did not show any simple relation between these two parameters. It is now clear that two pools of sterols exist in biological membranes. The most important is quantitative and is implicated in the regulation of membrane fluidity. The second could be implicated in the regulation of some membrane enzymes activities (SCHULER I. *et al.* 1991). There are now few evidences that ATPases are under this control (VENKEN M. *et al.* 1991). The implication in flowering process of these two phenomena is until now speculative.

However, sterols modifications found in spinach and in chenopodium could be due to a shunt of a precursor to another metabolic way. At this step, an important thing is to note that G proteins which are clearly implicated in the transduction of environmental signals require in most cases prenyl or farnesyl moieties for their anchorage at plasma membrane level (For review *see* GLOMSET J.A. *et al.* 1990). Another puzzling phenomenon has been demonstrated by DAFNA BAR-SAGI *et al.* (1986) which has been able to produce plasma membrane ruffling by an injection of ras, a G protein, in synchronized animal cell culture. A bulk of sterol and sequential protein prenylation and/or farnesylation has also been clearly correlated to different steps of the cell cycle (SEPP-LORENZINO L. *et al.* 1991). An increase of sterol content has been demonstrated in the apical meristem of spinach during the first hours of floral induction (CREVECOEUR M. *et al.* 1992, GARG V.K. *et al.* 1987). Some studies have also shown that the cell cycle in the meristem of *Silene armeria* was controlled by light and gibberellic acid and that this effect could be linked to flowering (BESNARD-WIBAUD C. *et al.* 1989).

Some experiments, described in the second part of this study, try to determine whether a direct or an indirect link exists between sterol content and plasma membrane modifications. The use of paclobutrazol, and principally its enantiomer 2R3R, inhibits the difference between plasmalemma thickness of induced and non induced plants. The enantiomer 2S3S, an inhibitor of gibberellin metabolism was without effect. Analysis in detail of these experiments shown that plasmalemma thickness of non induced plants was increased and that of induced plants decreased. Taking into account that at least one enzyme of gibberellin end metabolism was under light control (GIANFAGNA T. *et al.* 1983), this could be simply explained by a derivation of isoprene metabolism at an early step (BACH T.J. 1987, GOLSTEIN J.L. *et al.* 1990). This hypothesis that implicates a G

protein and mainly its anchorage at membrane level could be related to the fact that phospholipase C activity was different under light or dark conditions in spinach and in chenopodium leaves (MARTINEC J. *et al.* unpublished results). This signal pathway and its modulation could be directly under the phytochrome control, via a protein kinase (SINGH B.R. *et al.* 1990, DOSHI A. *et al.* 1992).

In conclusion, plasma membrane thickness and/or aspect could be used as early markers of the acquisition by the plants of their floral state. If this point is clearly established, we have now to explain its meaning in relation with the leaves induction and to consider the implications of this phenomena with the transmission of a floral stimulus. A relation could exist with ubiquitous transmembrane signal pathway and Ca^{2+} ions as signal transducer at cell level. A modification of the physico-chemical state of membrane which can be propagated throughout the whole plant to the apical meristem could explain the transmission of the flowering signal. This last aspect should not be neglected.

ACKNOWLEDGEMENTS

FIG. 1.

Effect of light and of gibberellic acid on leaves plasmalemma thickness of *Spinacia oleracea* (nm ± standard deviation). Plants received a total of 24-hrs light before plasmalemma isolation (1A). For gibberellic acid experiments, plants have been maintained under short day conditions and treated during three days. Control plants were sprayed with 0.5% MeOH (1B). SD: Plants maintained under short day conditions. LD: Plants maintained under long day conditions.

N°1A.

N°1 B.
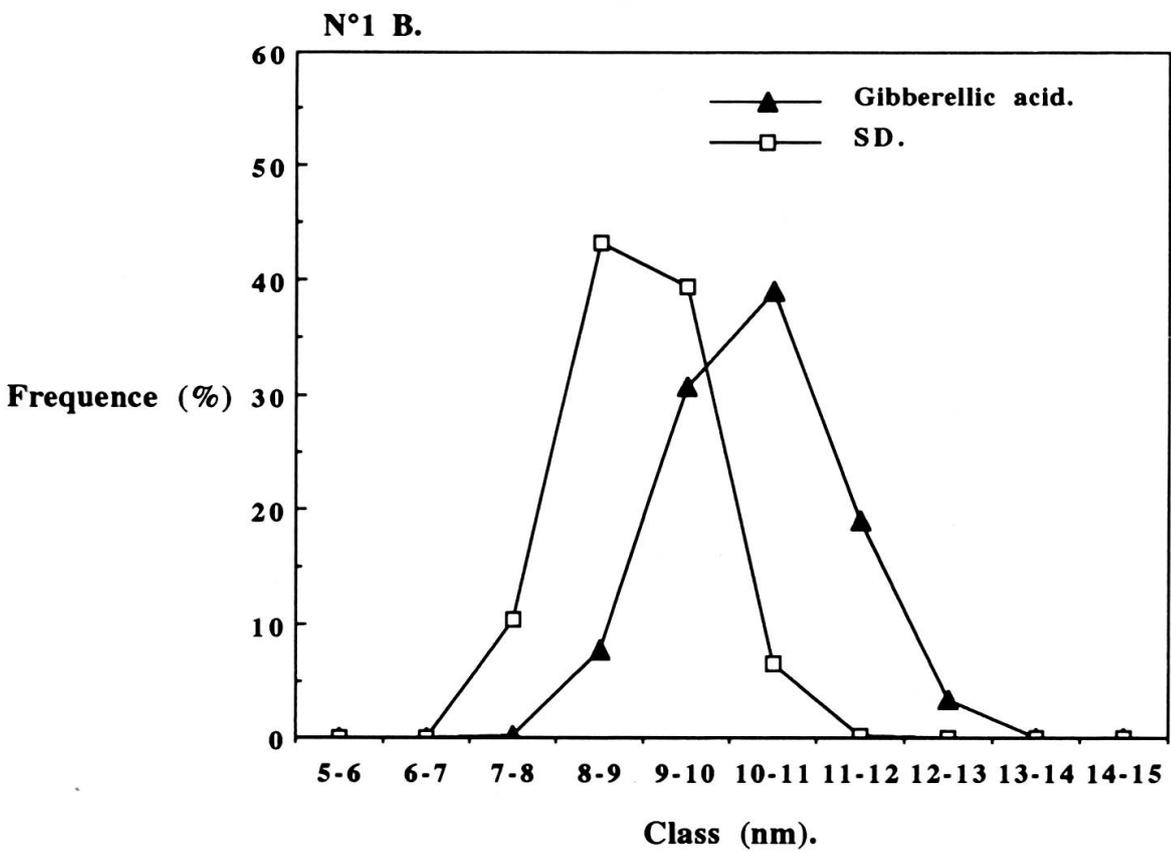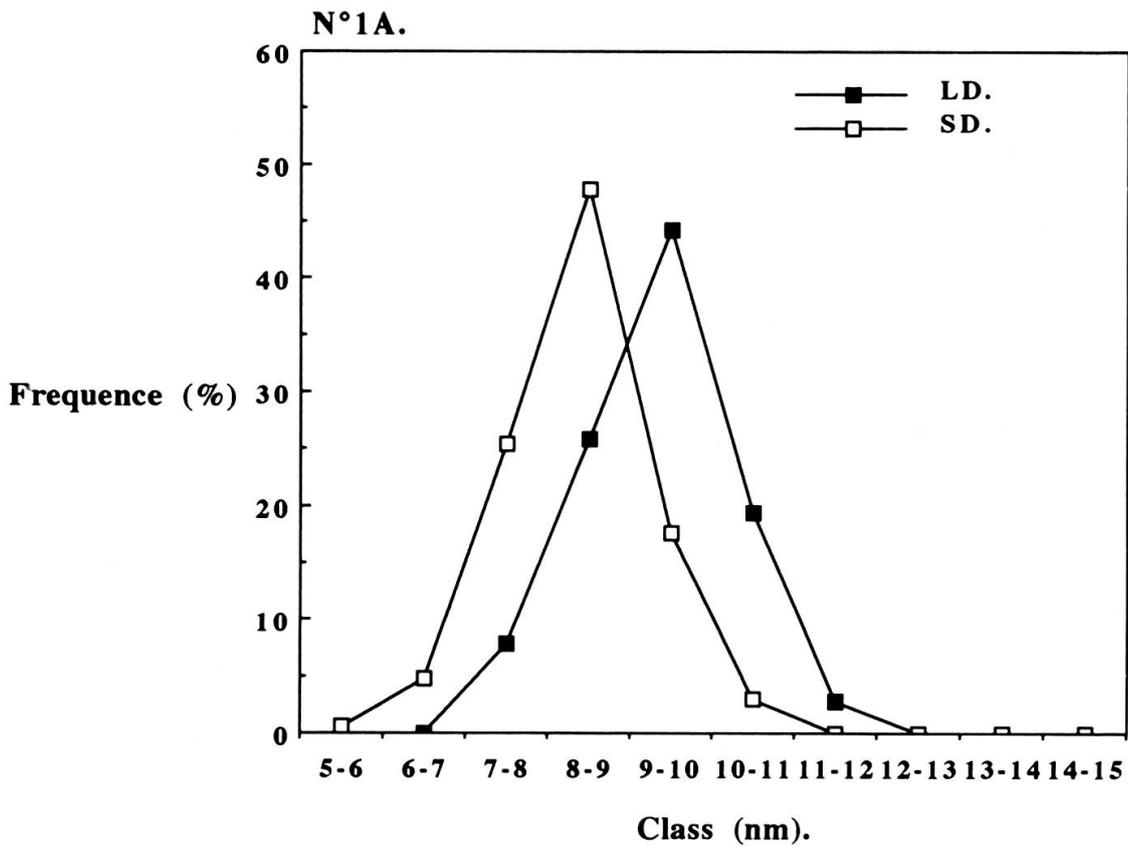
Experiment n°1.
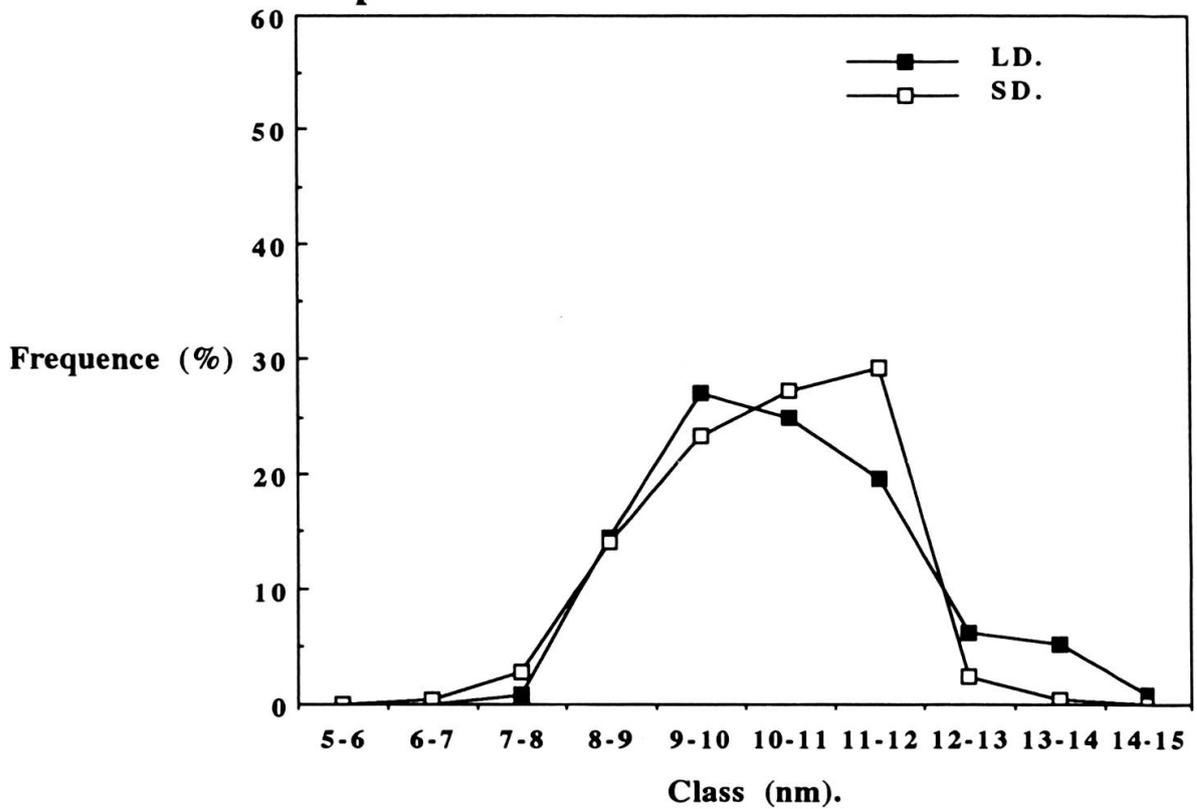
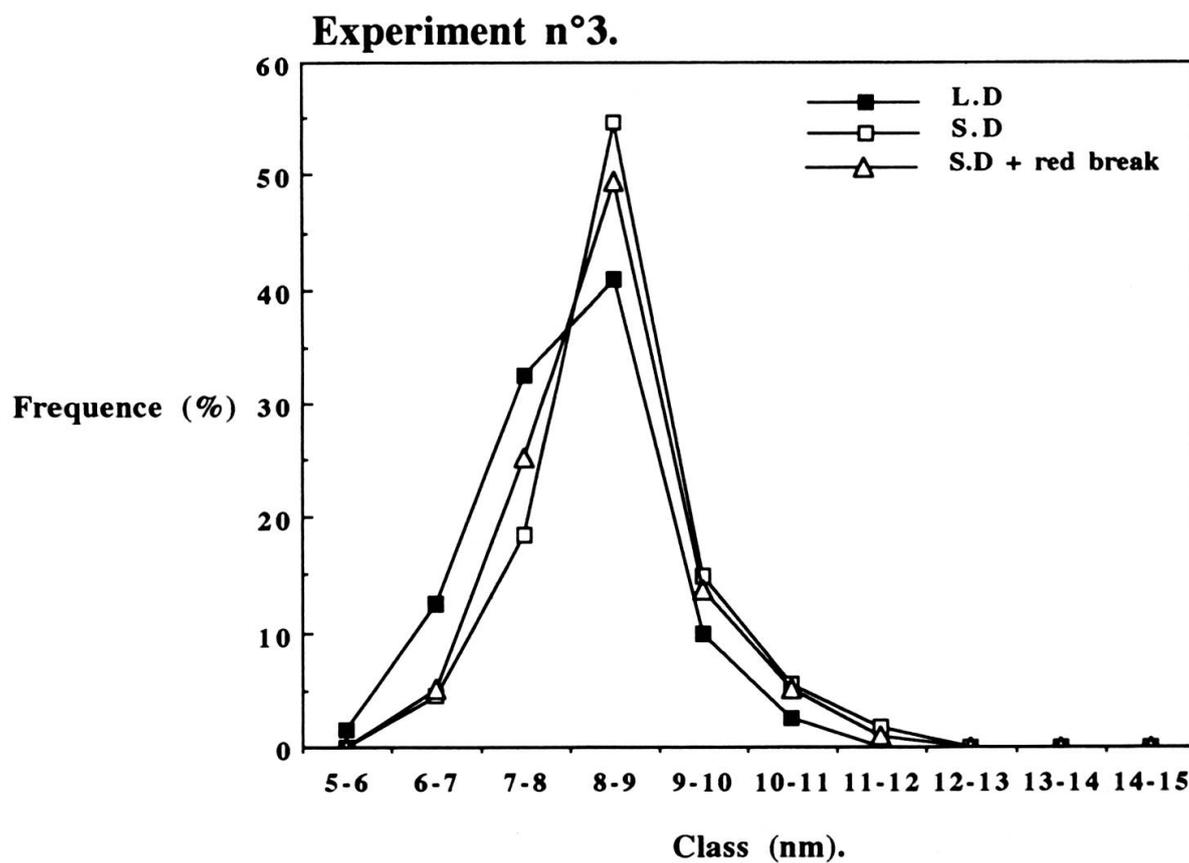## Experiment n°2.
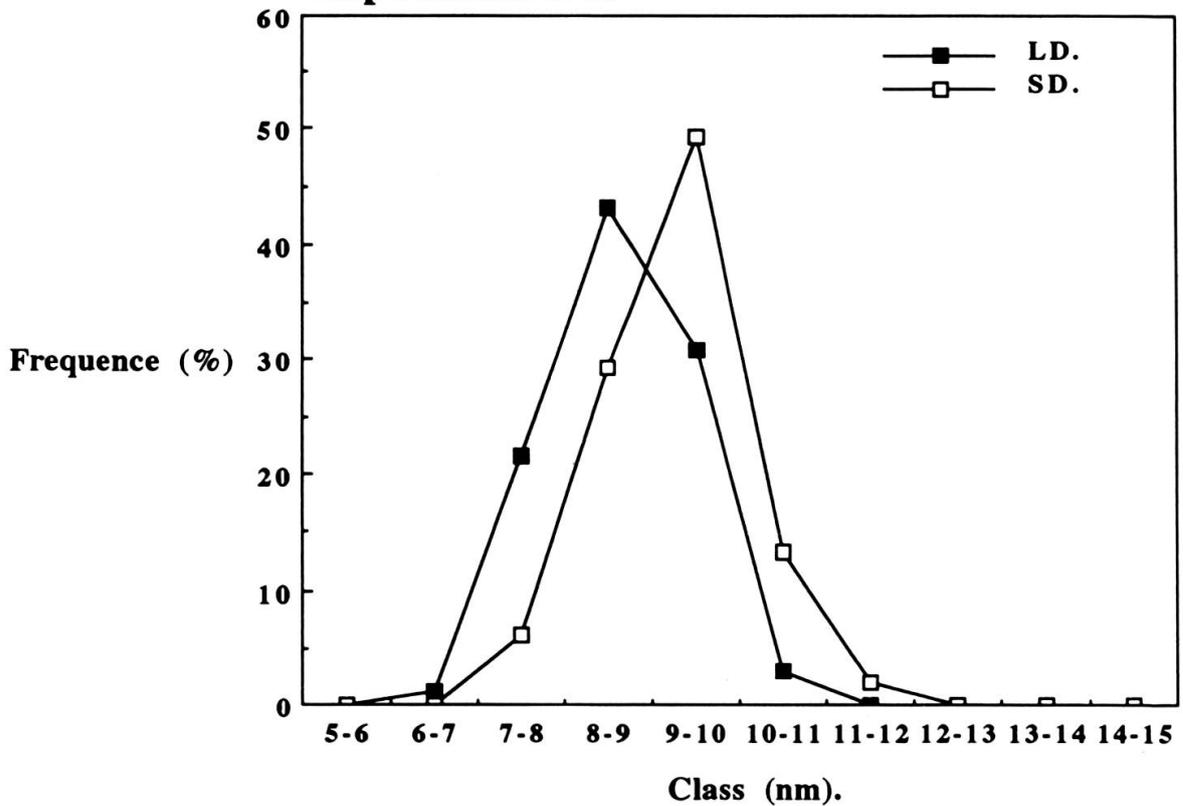
Experiment n°3.

FIG. 2. (Experiments n° 1, 2 and 3)

Effect of darkness on leaves plasmalemma thickness of *Chenopodium rubrum* (nm ± standard deviation). Control plants were maintained under long day condition and plasmalemma were extrated after a 12-hrs dark period. SD: Plants maintained under short day conditions. LD: Plants maintained under long day conditions.
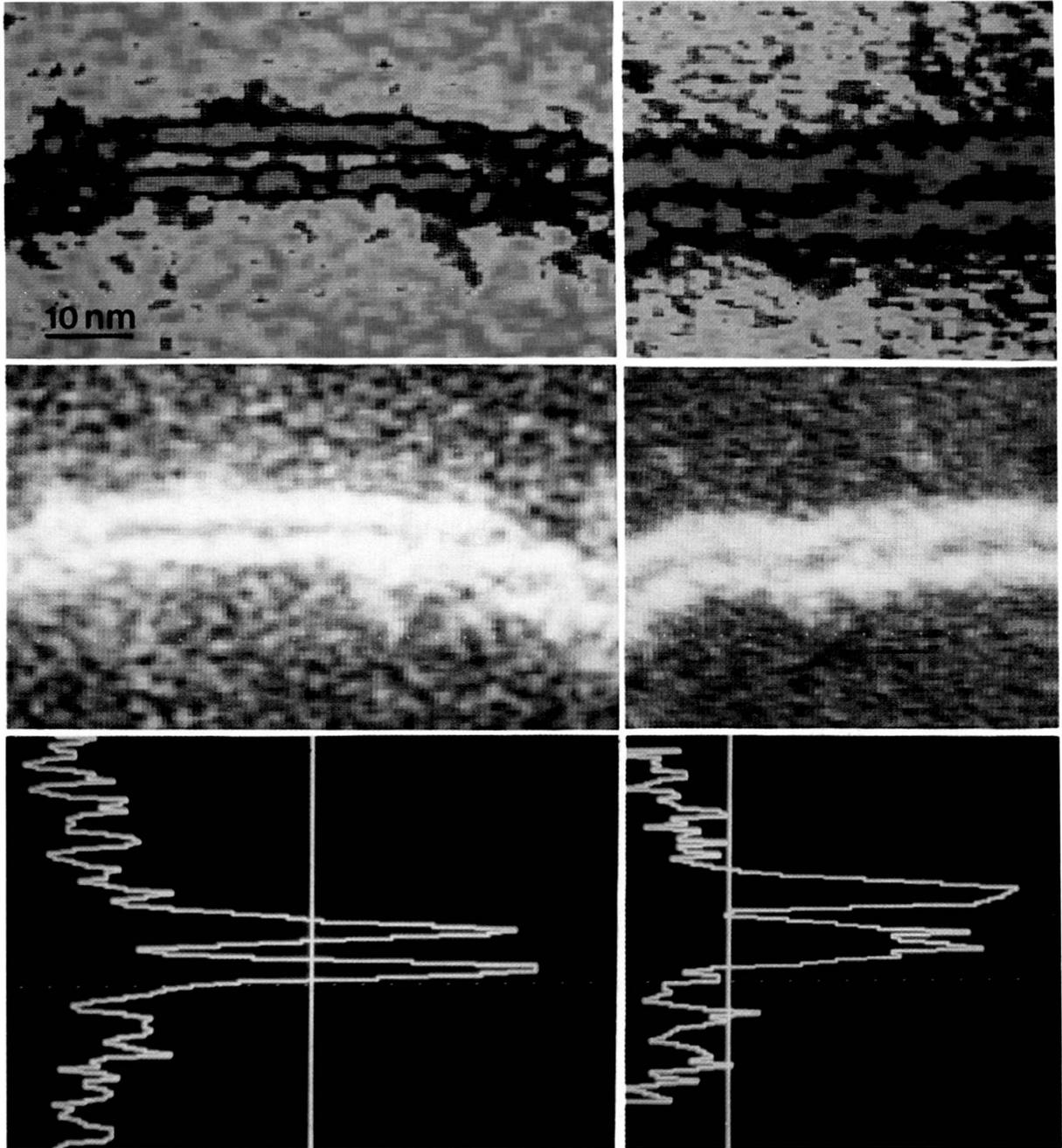
3A
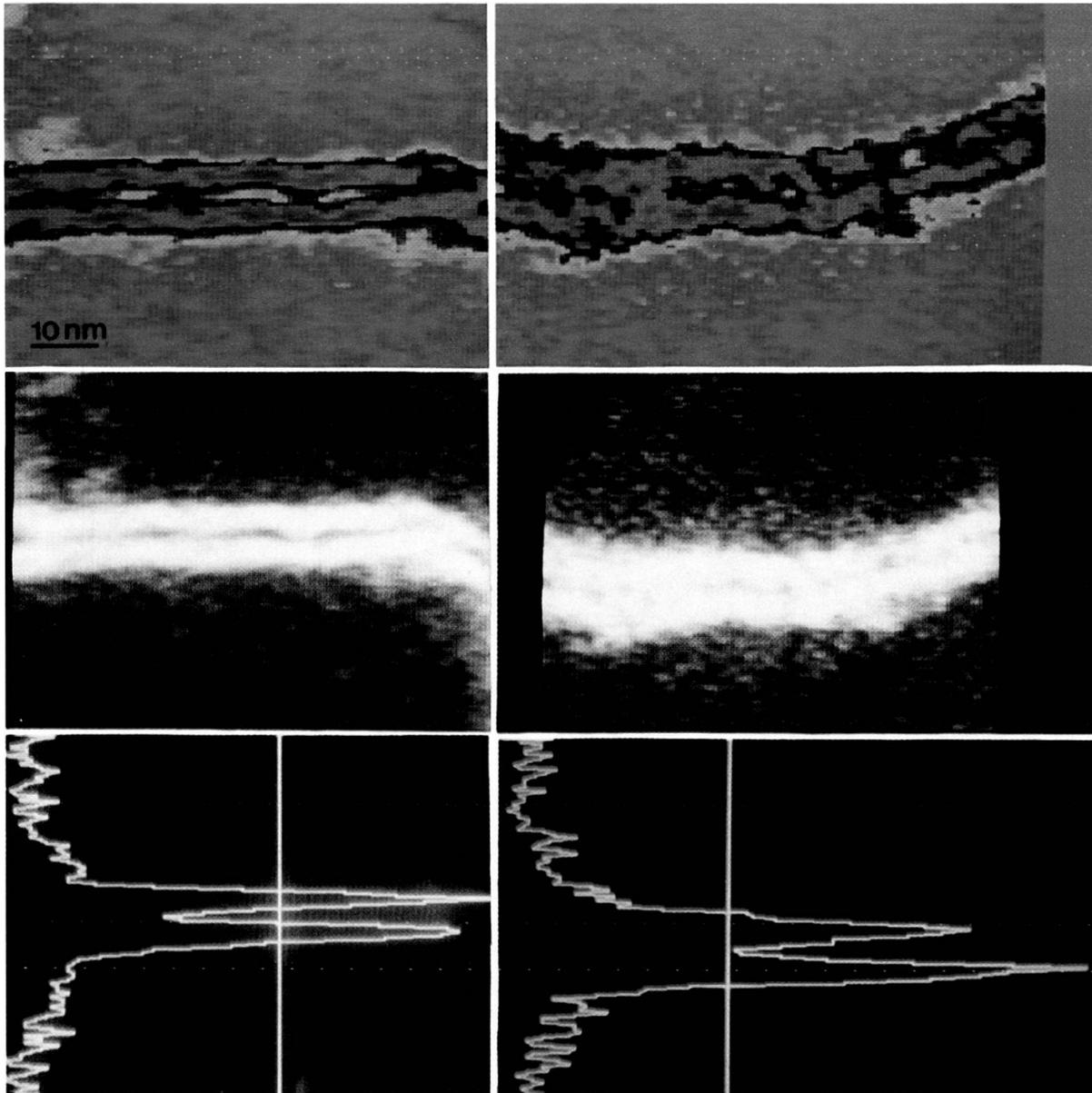
3B

FIGS 3A & 3B.

Portions of digitalized electron micrographs of plasmalemma from spinach (Fig. 3A) and from chenopodium (Fig. 3B) leaves. In both cases, pictures were given for vegetative (left pictures) and induced (right pictures) plants, in pseudocolor and in grey level. A densitometric trace was also given for each membrane portion.
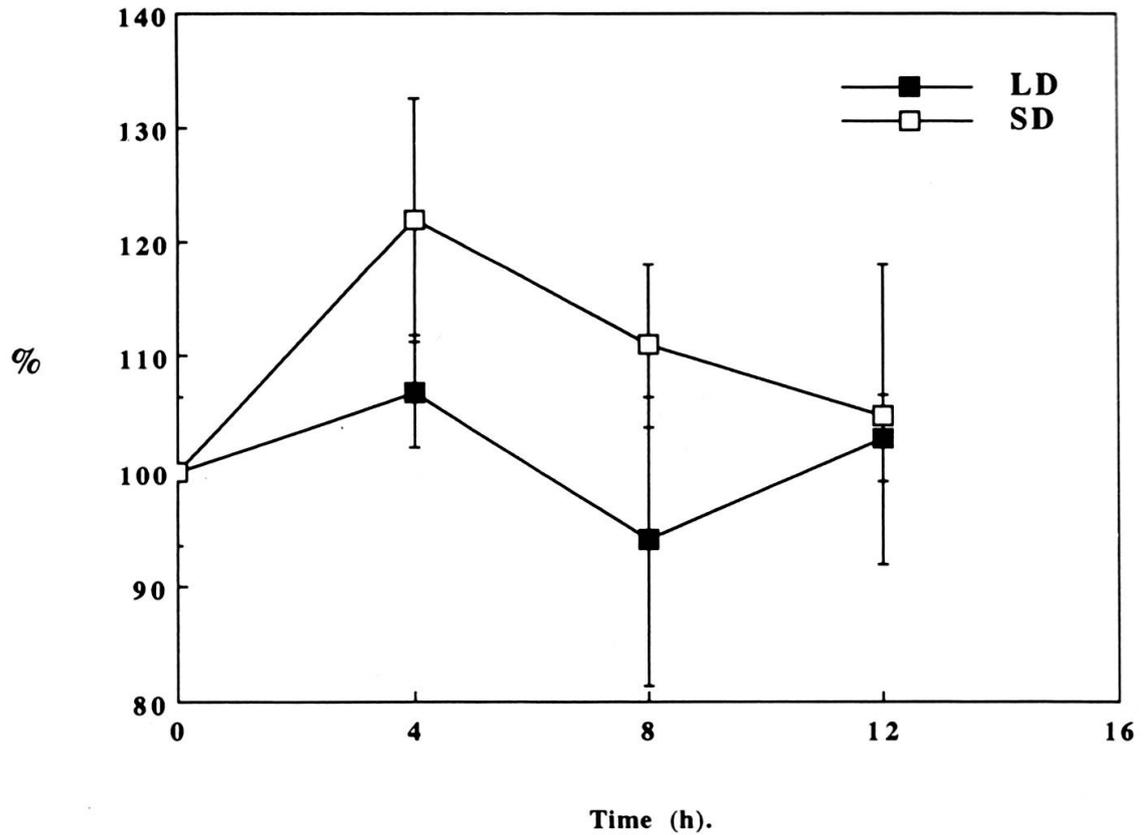
FIG. 4.

Modifications of total sterol content of chenopodium leaves plasma membrane during the first hours of inductive dark period (100%: start of the dark period). SD: Plants maintained under short day conditions. LD: Plants maintained under long day conditions.
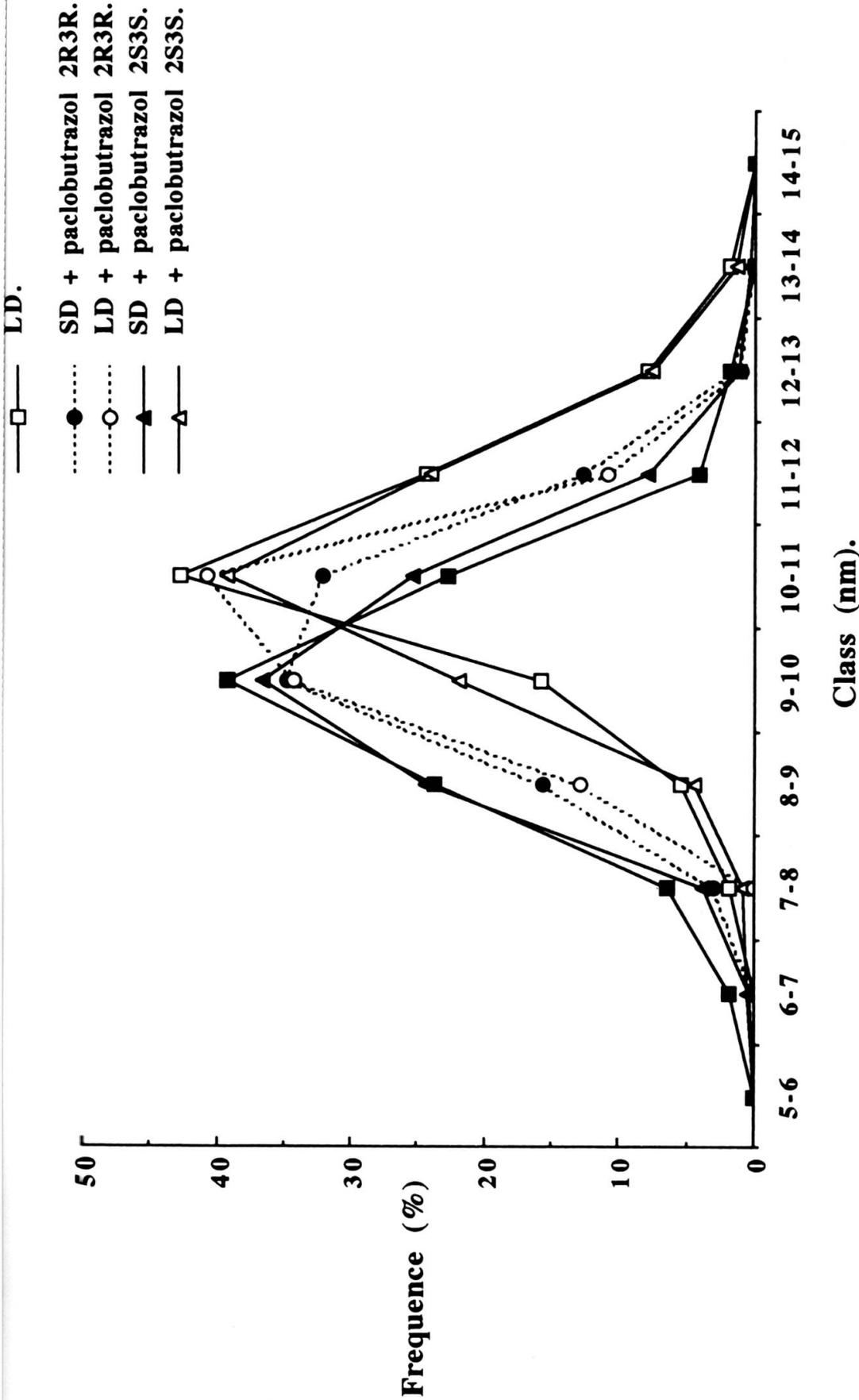
Fig. 5.

Effects on plasmalemma thickness (mm ± standard deviation) of spinach leaves of the differents isomers of paclobutrazol (2R3R; 2S3S). Plants were sprayed two times with 50 mg/l of paclobutrazol. Plasmalemma were isolated as described earlier after 24-hrs continuous light. SD: Plants maintained under short day conditions. LD: Plants maintained under long day conditions.

TABLE 1.

Plasma membrane thickness modifications during floral induction of *Spinacia oleracea* and *Chenopodium rubrum* (nm ± standard deviation). * Values statistically different from the control (Student t-test, P ≤ 0.005). ** Values statistically different from the control (Student t-test, P ≤ 0.025). Values indicated for spinach are representative of several experiments. SD: Plants maintained under short day conditions. LD: Plants maintained under long day conditions.

	mean ± s.d.	minimum	maximum	median	mode
Spinacia oleracea					
control (SD)	8.39 ± 0.86	5.82	11.04	8.34	8.26
LD	9.31 ± 0.88*	7.14	11.66	9.32	9.32
control (SD)	8.86 ± 0.74	7.00	11.20	8.90	9.00
AG₃	10.22 ± 1.00*	7.90	12.90	10.20	10.20
Chenopodium rubrum					
experiment n° 1					
control (LD)	7.67 ± 0.81	5.36	9.76	7.60	7.60
SD	8.29 ± 0.80**	6.21	10.39	8.27	8.27
exeriment n° 2					
control (LD)	10.21 ± 1.10	6.20	13.24	10.32	10.32
SD	10.44 ± 1.40	7.36	15.24	10.32	9.64
experiment n° 3					
control (LD)	8.05 ± 0.94	5.64	10.96	8.12	8.76
SD	8.51 ± 0.87**	6.40	11.28	8.40	8.76
SD + red break	8.35 ± 0.88	6.40	11.24	8.16	8.12

TABLE 2.

Effects of differents inhibitiors of sterols and gibberellins metabolisms on plasmalemma thickness of spinach leaves (nm ± standard deviation). SD: plants maintained under short day conditions. LD: Plants maintained under long day conditions. 2S3S: Enantiomer 2S3S of paclobutrazol, an inhibitor of gibberellin metabolism. 2R3R: Enantiomer 2R3R of paclobutrazol, an inhitor of sterol metabolism. *Values statistically different from the control (Student t-test, P ≤ 0.005).

	mean ± s.d.	minimum	maximum	median	mode
Experiment with paclobutrazol					
SD (control)	9.40 ± 1.10	6.02	12.55	9.42	9.07
LD	10.60* ± 1.00	7.7	13.35	10.50	10.42
SD + 2S3S	9.60 ± 1.00	6.35	13.65	9.52	9.52
LD + 2S3S	10.60* ± 1.00	6.47	13.67	10.55	10.90
SD + 2R3R	9.90 ± 1.00	7.27	12.22	9.95	9.42
LD + 2R3R	10.00 ± 0.85	8.12	12.65	10.00	9.52

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