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# INFLUENCE OF ELEVATED $CO_2$ ON CELL ELONGATION AND STOMATAL DENSITY IN WHITE MUSTARD (SINAPIS ALBA)

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## ABSTRACT

*The effects of carbon dioxide on stomatal density, stomatal index, stomatal size, and epidermal cell density were examined in white mustard (Sinapis alba L.) using scanning electron microscopy. Stem diameter, and the length and width of cortical parenchyma cells were examined using light microscopy. Stem length was also measured. Plants were grown in growth chambers maintained at 20$^{o}$C and at one of three levels of carbon dioxide (350, 700, and 1400±50 ppm). Seedlings were provided with enough light to induce the unfolding of the cotyledons but not enough to result in any significant photosynthesis (PAR = 45 mmol $m^{-2}$ $s^{-1}$ for 10 minutes per day). The results indicated that stem elongation, lengths of both guard and subsidiary-cells, and lengths of parenchyma cells were all significantly increased by elevated carbon dioxide. In contrast, stomatal density and epidermal cell density were decreased for both cotyledon surfaces. Level of carbon dioxide had no effect on stem diameter, diameter of parenchyma cells or on stomatal index in the studied species. The results suggest that elevated carbon dioxide increases longitudinal cell expansion and that this has various consequences such as a decrease in stomatal density and increases in stem height. Further, the effect of carbon dioxide on cell expansion is independent of its effects on photosynthesis.*

***Key words:*** *Sinapis alba, elevated CO2 ,stomatal density, stomatal size and scanning electron microscopy.*

## INTRODUCTION

There is no doubt that atmospheric levels of carbon dioxide have been rising and that this rise is chiefly due to fuel combustion and deforestation. $CO_2$ levels in the atmosphere now stand at 387 parts per million (ppm), up almost 40% since the industrial revolution. The current concentration is higher than at any time in the past 650,000years (IPCC, 2007), and it is predicted that $CO_2$ levels will double before the end of this century (Cao & Woodward, 1998). Therefore, researchers have shown a great deal of interest in the effects of CO2 on plant growth (Royer, 2001). Information on plant responses to CO enrichment may help to forecast consequences for plant productivity, community composition, and ecosystem structure (Körner, 2000; Norby, 2004).

It is generally accepted that the primary effects of increasing $CO_2$ concentration are on photosynthesis and stomatal conductance: where photosynthesis is increased and stomatal conductance is decreased. Increases in photosynthesis are due to the beneficial

effects of $CO_2$ concentration on carboxylation efficiency and the consequent decrease in photorespiration. Decreases in stomatal conductance are due -to the effect of internal $CO_2$ upon stomatal closure (Bazzaz, 1990). This decline in stomatal conductance leads to a reduction in water loss and enhances plant water use efficiency (defined as the number of units of $CO_2$ fixed per unit of water lost).

Although the major effects of elevated $CO_2$ on plants are on photosynthesis and stomatal conductance, it modifies a broad range of plant processes including many aspects of development, such as: leaf morphology and anatomy, biomass allocation, time of flowering, rate of senescence, branching patterns and stem elongation (Strain and Cure, 1985; Eamus and Jarvis, 1989; Bazzaz, 1990; Zebian and Reekie, 1998). The effects of $CO_2$ upon development can be complicated, for example, depending upon species and environmental conditions, elevated $CO_2$ may delay, hasten, or have no effect on time of flowering (Reekie and Bazzaz, 1991; He and Bazzaz, 2003; Johnston and Reekie, 2008). The mechanistic basics for these direct effects of $CO_2$ on phenology and development are still unclear (Johnston and Reekie, 2008). In general, developmental effects such as these are often considered to be secondary consequences of the effect of $CO_2$ on photosynthesis and stomatal conductance and therefore on growth (e.g. He and Bazzaz, 2003). However, the effects of $CO_2$ on phenology are not necessarily correlated with its effects on growth (Reekie and Bazzaz, 1991). Furthermore, it has been shown that $CO_2$ can affect stem elongation in complete darkness (i.e. in the absence of any of its effects on photosynthesis or growth) (Zebian and Reekie, 1998).

It has long been known that level of $CO_2$ can affect stomatal density (O'Leary and Knecht, 1981; Woodward, 1987, Woodward and Bazzaz, 1988, Rowland-Bamford *et al.,* 1990; Radoglou and Jarvis, 1990; Radoglou and Jarvis, 1992; Woodward, 1992; Ceulemans *et al.,* 1995). In many cases it has been shown that this effect is related to a positive effect of $CO_2$ on cell size in that although elevated $CO_2$ may decrease the number of stomata per unit area, stomatal index (number of stomata/number of epidermal cells) is often unaffected (Radoglou and Jarvis, 1990; Radoglou and Jarvis, 1992; Ryle and Stanley, 1992; Malone *et al.,* 1993; Lauber and Korner, 1997).

Given that the effect of $CO_2$ on cell size may be related to its positive effect on photosynthesis and growth, its effect on stomatal density may also be related to these effects. Therefore, the present study was undertaken to determine if carbon dioxide can affect stomatal density in the absence of any significant photosynthesis.

## MATERIALS AND METHODS

White mustard *(Sinapis alba L.)* was grown at three levels of carbon dioxide. Seedlings were provided with enough light to induce the unfolding of the cotyledons but not enough to result in any significant photosynthesis. We examined the effect of these treatments on stomatal density, stomatal index, stomatal size, and epidermal cell density in the studied species. Stem height and diameter*, and length and width of stem parenchyma cells

were also measured to determine if the effect of $CO_2$ on epidermal cells was similar to its effect on stem cells. We hypothesized that if $CO_2$ can affect these anatomical traits in the absence of any effects on growth, this will indicate that $CO_2$ affects these traits through directly influencing developmental patterns. We also hypothesized that if stomatal index does not change with level of carbon dioxide, this will indicate that both the reported decreases in stomatal density can be attributed to simple increases in cell size and therefore decreases in number of cells per unit area.

**Carbon Dioxide Fumigation System:**

The seedlings were exposed to the different $CO_2$ concentrations (350, 700, and 1400 ppm) using a flow through fumigation system. The fumigation chambers (0.75 L) consisted of an 11 cm length of a 10 cm diameter PVC pipe with an opaque cap on one end and a removable transparent plexiglass cover on the other. Each chamber had a 0.5 cm diameter inlet tube on one side and a 0.7 cm diameter outlet tube on the other. The outlet tube was connected to a 30 cm length of Tygon tubing to minimize back diffusion into the chamber. The inlet tube of each chamber was coupled to a humidified air supply system which provided a flow of 1L per minute per chamber. The air was obtained from a pressure regulated compressed air source that sampled air from outside the building at a height of about 2m above ground level. The 350 ppm $CO_2$ treatment was unmodified ambient air. The 700 and 1400 ppm $CO_2$ treatments were obtained by bleeding pure $CO_2$ into the atmospheric air source via needle valves. The level of $CO_2$ in the various airstreams was determined using a Li-Cor model 6250 infrared gas analyser. All fumigation chambers were placed in the same controlled environment chamber (Conviron model E15) which maintained a constant temperature of 20$^{o}$C.

**Plant culture:**

Fifteen fumigation chambers were used, five at each of three carbon dioxide levels. The studied species was grown from seed in 50 ml vials containing 0.6% agar. A wick was placed in the agar with both ends protruding through holes that were drilled in the sides of the vials. The vials were then placed in the fumigation chambers that contained about 60 ml distilled water. The wicks absorbed water thus keeping the agar surface moist. Ten seeds were evenly spread upon the surface of the agar in each vial. There was a total of 30 seedlings per chamber (i.e. 3 vials per chamber). Seedlings were harvested after 6 days post germination.

**Experimental Measurements:**

Seedlings were harvested, stem height recorded and samples stored in formalin- aceto-alcohol (FAA) solution. To prepare samples for Scanning Electron Microscopy (SEM), one cotyledon per chamber per treatment was fixed in 2.5% Glutaraldehyde in PO4 buffer for 2-4 hours. After 3 successive washes in 0.1 M PO4 buffer, the samples were then postfixed in 1% $OsO_4$ in 0.1 M $PO_4$ buffer for 1 hour. The samples were washed twice with 0.1 M PO4 buffer and dehydrated through a graded acetone series (15, 30, 50, 70, 80, 90, 95%) for 10 minutes per concentration. Three changes of 100% acetone completed the process. Critical point drying from the acetone followed. The cotyledons were mounted on stubs and sputter coated with 15-20 nm gold-

palladium. The cotyledon surfaces were viewed at 15 KV on a JSM 25S. Stomatal density measurements were recorded by counts made on screen. A field of view on the cotyledon surface was chosen randomly (excluding fields that contained the midrib). The total number of stomata (per field of view at 450X) was counted. This procedure was repeated for both the lower and upper surfaces for each cotyledon.

Lengths and widths of both guard and subsidiary cells were measured from Scanning electron micrographs (at 1000X). All stomata visible on the photograph were measured. These photographs were also used to measure epidermal cell density by counting the total number of the epidermal cells visible on the photograph. Data from one cotyledon per chamber were recorded. Stomatal index was calculated as follow: number of stomata per unit area / (number of stomata + number of epidermal cells per unit area)x100.

Light microscopy was used to measure both the length and width of parenchyma cells and the diameter of the stem. FAA fixed stems were cut 0.5 cm below the cotyledons into sections not longer than 0.5 cm. The sections were dehydrated using a Tertiary Butyl Alcohol (TBA) series followed by infiltration in paraffin wax. The samples were then embedded in hot paraffin. The resulting paraffin blocks were trimmed and mounted onto wooden blocks. Blocks were sectioned (30μm) both transversely and longitudinally and sections mounted on glass slides. Observations were made at 16X and the diameter of the stems recorded from the transverse sections. For measurements of length and width of cortical parenchyma cells, the longitudinal sections were stained with fast green. Observations were made of cell length and width at 40X. A row of cells located midway between the epidermis and the edge of the vascular cylinder was chosen for measurement. From this row the dimensions of 5 adjacent cells per stem were recorded with 5 stems per treatment (i.e. one stem /chamber).

**Statistical analysis:**

These data were analyzed using one-way ANOVA for normally distributed variables with $CO_2$ as the independent variable and Kruskal-Wallis non-parametric tests for non-normally distributed variables. All analyses were done using Sigma Plot (Version 11) for a personal computer Royer, (2001)..

## RESULTS AND DISCUSSION

The level of carbon dioxide had no effect on stem diameter or width of stem parenchyma cells (fig 1A, table 1). However, it had a pronounced effect on stem height and length of stem parenchyma cells (fig 1B, table 1). There was an approximately linear increase in cell length with carbon dioxide, while, the greatest change in stem height was between the 700 $\mu l\ l^{-1}$ and 1400 $\mu l\ l^{-1}$ treatments.

Stomatal frequencies were lower on the upper cotyledon surface. Stomatal and epidermal cell density (fig 2 and table 1) were significantly decreased by elevated carbon dioxide on both the upper and lower cotyledon surfaces. There was an approximately linear decrease in stomatal density with carbon dioxide. There was an approximately linear decrease in epidermal cell density with carbon dioxide on the lower cotyledon surface. On the

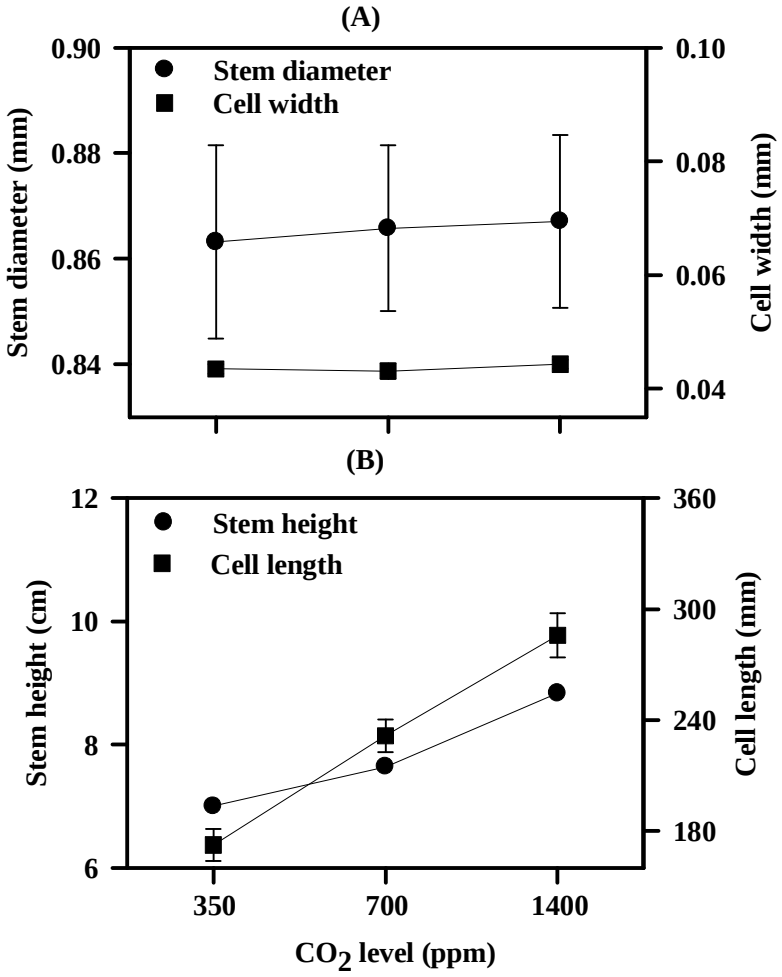


**Fig. 1.** The effect of $CO_2$ on stem diameter and cell width (A) and on stem height and cell length (B) in *Sinapis alba*. Error bars represent ±1SE. Where bars are not shown, these were either smaller than the size of the symbol or the data were not normally distributed.

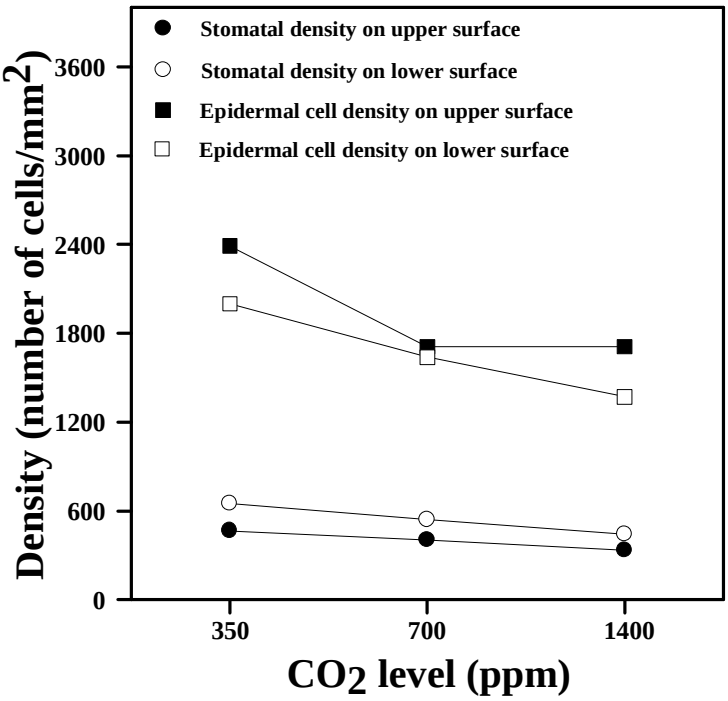


**Fig. 2.** The effect of $CO_2$ on stomatal and epidermal cell density of S*inapis alba*. Error bars represent ±1SE. Where bars are not shown, these were either smaller than the size of the symbol or the data were not normally distributed.

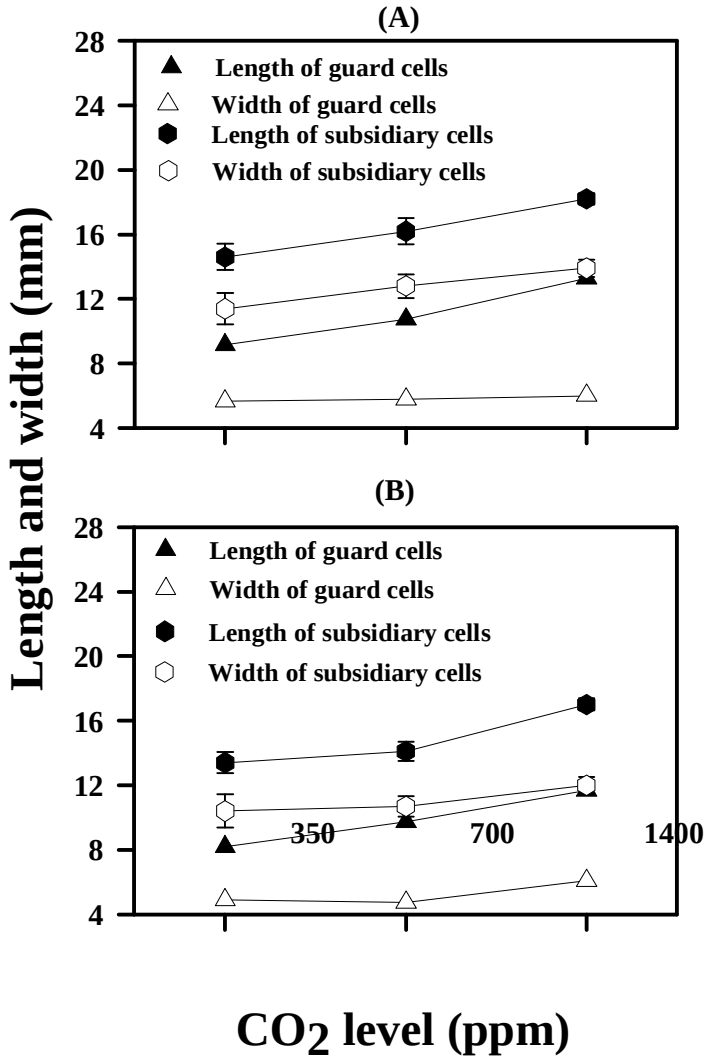


**Fig. 3.** The effect of $CO_2$ on stomatal size in *Sinapis alba* on both the upper (A) and the lower cotyledon surfaces (B). Error bars represent ±1SE. Where bars are not shown, these were either smaller than the size of the symbol or the data were not normally distributed.

**Table 1.** The level of significance for the effect of carbon dioxide on anatomical and morphological characteristics of *Sinapis alba*.

| Variable | Level of significance |
|---|---|
| Stomatal density | |
| Upper surface | 0.0003* |
| Lower surface | <0.0001* |
| Epidermal cell density | |
| Upper surface | 0.0033† |
| Lower surface | <0.0001* |
| Length of guard cell | |
| Upper surface | <0.0001* |
| Lower surface | 0.0019† |
| Width of guard cell | |
| Upper surface | 0.557* |
| Lower surface | 0.0619* |
| Length of subsidiary cell | |
| Upper surface | 0.0110* |
| Lower surface | 0.0016* |
| Width of subsidiary cell | |
| Upper surface | 0.102* |
| Lower surface | 0.313* |
| Stomatal index | |
| Upper surface | 0.415* |
| Lower surface | 0.317* |
| Stem height | 0.0019† |
| Stem diameter | 0.986* |
| Parenchyma cell length | <0.0001* |
| Parenchyma cell width | 0.684* |

Note: *, One-way ANOVA and †, Kruskal-Wallis test.

upper cotyledon surface, the greatest change in epidermal cell density was between the 350 $\mu l\ l^{-1}$ and 700 $\mu l\ l^{-1}$ treatments. Stomatal index (defined as the ratio of stomatal cells to epidermal cells expressed as a percentage) was not affected by level of carbon dioxide in the studied species.

Level of carbon dioxide caused a significant increase in the length of both guard and subsidiary cells (fig 3 and table 1). For the most part, there was a linear increase in the length of both guard and subsidiary cells with carbon dioxide. However, in the case of subsidiary cells on the lower surface the greatest change was between the 700 $\mu l\ l^{-1}$ and 1400 $\mu l\ l^{-1}$ treatments.

The present results confirm earlier studies that have found a poor relationship between the effect of carbon dioxide upon growth and its effect upon morphology and phenology (Reekie and Bazzaz, 1991; Zebian and Reekie, 1998). For instance, in our study, the fact that carbon dioxide affected stem elongation and stomatal density at extremely low light levels (i.e. in the absence of any of its effects on photosynthesis) confirms that the effect of $CO_2$ on development is not necessarily related to its effect on photosynthesis.

Our results suggest that elevated CO2 increases cell expansion and that this has various consequences such as a decrease in stomatal density and increases in stem height. The fact that stomatal index does not change with level of $CO_2$ indicates that the decrease in stomatal density at elevated $CO_2$ can be entirely attributed to simple increases in cell size and therefore decreases in numbers of cells per unit area. Similarly, the 26% increase in height between 350 and 1400 ppm $CO_2$ can be more than accounted for by the 66% increase in length of stem cells. The effect of $CO_2$ on these two divergent aspects of plant development (i.e. stomatal density and stem elongation) appears to be the result of the effect of $CO_2$ on a single process, cell expansion.

Greater cell expansion, which occurs at higher $CO_2$ levels, has been attributed to the increased osmotic potential of leaf cells associated with their higher total carbohydrate content, which causes the cells to absorb more water and thus enlarge (Madsen, 1973). However, our observations suggest that the effect of $CO_2$ on cell size was not dependent on its effects on photosynthesis. Plants were not provided with sufficient light to result in any significant photosynthesis. Therefore, the increase in cell size cannot be attributed to the positive effect of elevated $CO_2$ on carbohydrate accumulation. This raises the question of how $CO_2$ affects cell expansion if not through effects on photosynthesis.

One possible mechanism is an interaction between $CO_2$ and plant growth regulators such as ethylene. At high concentrations (50,000-100,000 ml-1), $CO_2$ appears to interfere with the role of ethylene by affecting its synthesis. High levels of $CO_2$ decrease the conversion of ACC (1-Aminocyclopropan-1-Carboxylic acid) to ethylene by acting as a competitive inhibitor (Cheverry et al. 1988). If the levels of $CO_2$ used in our study decreased ethylene production, this could explain the increased stem elongation at high $CO_2$ levels as ethylene inhibits stem elongation probably by

inhibiting cell elongation (Camp and Wickliff, 1988; Eisinger, 1983). This could explain the effect of $CO_2$ on the size of epidermal cells. Although the levels of carbon dioxide used in our study were much lower than those generally reported to inhibit ethylene production, at least one study has shown that a doubling of ambient $CO_2$ levels can affect ethylene production in some species (Lu and Kirkham, 1992). Another possible mechanism by which $CO_2$ may affect cell expansion is an interaction between $CO_2$ and the developmental pathways responsible for sensing and transmitting light signals. Song et al. (2008) grew 10 mutants of *Arabidopsis thaliana* deficient in particular loci in the pathway responsible for sensing and measuring changes in photoperiod, as well as the wild type, under short and long days at either ambient or elevated $CO_2$. Elevated $CO_2$ interacted with both the sensing and the subsequent transduction of light signals. The direction and magnitude of the effects varied with photoperiod.. An interaction between phytochrome (one of the light sensors involved in photomorphogenic responses) and $CO_2$ has been suggested by several previous studies to explain the contrasting effects of $CO_2$ on flowering and development under long versus short day conditions (Purohit and Tregunna, 1974; Hicklenton and Jolliffe, 1980; Reekie *et al.,* 1994; Reekie *et al.,* 1997; Johnston and Reekie, 2008). Further, Zebian and Reekie (1998) suggest the contrasting effects of $CO_2$ on stem elongation in darkness versus light may also be related to an interaction with phytochrome. The effects of phytochrome on stem elongation have been shown to be primarily the result of changes in cell expansion, though in some cases cell division is also affected (Gaba and Black, 1983). Therefore an interaction between $CO_2$ and phytochrome might explain the effects of $CO_2$ on cell expansion observed in the present study.

A third possible mechanism by which $CO_2$ may affect cell expansion is via acidification (i.e. the so called acid growth theory). This theory states that auxin (indole-3-acetic acid, IAA) initiates an active acidification mechanism, possibly a membrane bound proton pump, so that pH of the wall solution decreases. Consequently, this lower pH activates wall-loosening enzymes, the wall is loosened, and cell enlargement takes place (Rayle and Cleland, 1970). When $CO_2$ dissolves in water it forms carbonic acid, which dissociates into bicarbonate and hydrogen ions, which in turn lower the pH. This acidic pH would promote cell enlargement. It has in fact been suggested that the effect of IAA on cell expansion is mediated through this $CO_2$ effect. Sloane and Sadava, (1975) suggest that IAA may increase cell respiration which in turn releases $CO_2$ and lowers the pH of the cell wall. However, it is unclear whether the levels of $CO_2$ used in the present study would be high enough to lower the pH of the cell wall sufficiently. At least one previous study has shown that a $CO_2$ concentration of 5% is required to induce significant cell elongation (Mer and Richards, 1950). However, different studies have shown different values for the pH required to initiate cell wall loosening and extension growth. These values range from 6-4. Such variation might explain the contrasting effects of $CO_2$ on different species, different plant tissues and different cell types.

## CONCLUSION

Based on the above mentioned pattern results it could be suggested that elevated carbon dioxide increases longitudinal cell expansion and that this has various consequences such as a decrease in stomatal density and increases in stem height. Further, the effect of carbon dioxide on cell expansion is independent of its effects on photosynthesis.

It is important to understand why $CO_2$ affects developmental patterns if we are going to accurately predict the effect of changes in the concentration of atmospheric carbon dioxide on plant communities. The present study is a step in that direction in that it demonstrates that $CO_2$ can affect both cell elongation and stomatal density independent of any of its effects on photosynthesis, but, clearly more work is required to elucidate the mechanism (s) by which $CO_2$ modifies developmental patterns.

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# الملخص العربى
# تأثير التركيزات المرتفعة من ثانى أكسيد الكربون على تمدد الخلية وكثافة الثغور فى الخردل الأبيض

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قسم النبات – كلية الآداب والعلوم جامعة عمر المختار طبرق – ليبيا

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يهدف هذا البحث نحو دراسة تأثير ثانى أكسيد الكربون على كل من كثافة الثغور، معامل الثغور، حجم الثغور وكثافة خلية البشرة فى نبات الخردل الأبيض، وذلك باستخدام المجهر الإلكترونى الماسح، كما تم تعيين قطر الساق وطول وعرض الخلية البرنيشيمية اللحائية باستخدام المجهر الضوئى، هذا بالإضافة إلى قياس طول الساق، وقد تنمية النبات فى غرف النمو المثبتة عند ٥٢٠م، وعند أحد مستويات ثانى أكسيد الكربون الثلاثة وهى (٣٥٠، ٧٠٠، ١٤٠٠ + ٥٠ جزء فى المليون). لقد زودت البادرات بقدر من الإضاءة بهدف تحفيز تفتح الفلقات إلا أنه ليس بالقدر الكافى لقيام البادرات بعملية البناء الضوئى المفيد، وذلك لكل عشر دقائق لكل يوم، وقد أظهرت النتائج إن إستطالة الساق وأطوال الخلايا الحارسة البرنشيمية قد ازدادت بشكل ملموس بزيادة تركيز ثانى أكسيد الكربون، وذلك على نقيد ماحدث لكثافة الثغور وخلايا البشرة التى تقلصت على كل من سطحى الفلقة، كما تبين على ضوء هذه الدراسة أيضاً إن مستويات ثانى أكسيد الكربون هذه لم تبدى أى تأثير على قطرى الساق والخلايا البرنشيمية أو حتى معامل الثغور فى هذا النوع قيد الدراسة، وقد برهنت النتائج المتحصل عليها قد برهنت على إنه كلما زاد تزكيز ثانى أكسيد الكربون يصحبه تمدد فى الخلية الطولية، الأمر الذى يترتب عليه عوامل أخرى كنقص كثافة الثغور وزيادة طول الساق، إضافة إلى أن تأثير ثانى أكسيد الكربون على التمدد الخلوى لايعتمد بدوره على تأثيره إزاء عملية البناء الضوئى.

JOESE 5

# INFLUENCE OF ELEVATED $CO_2$ ON CELL ELONGATION AND STOMATAL DENSITY IN WHITE MUSTARD (SINAPIS ALBA)

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