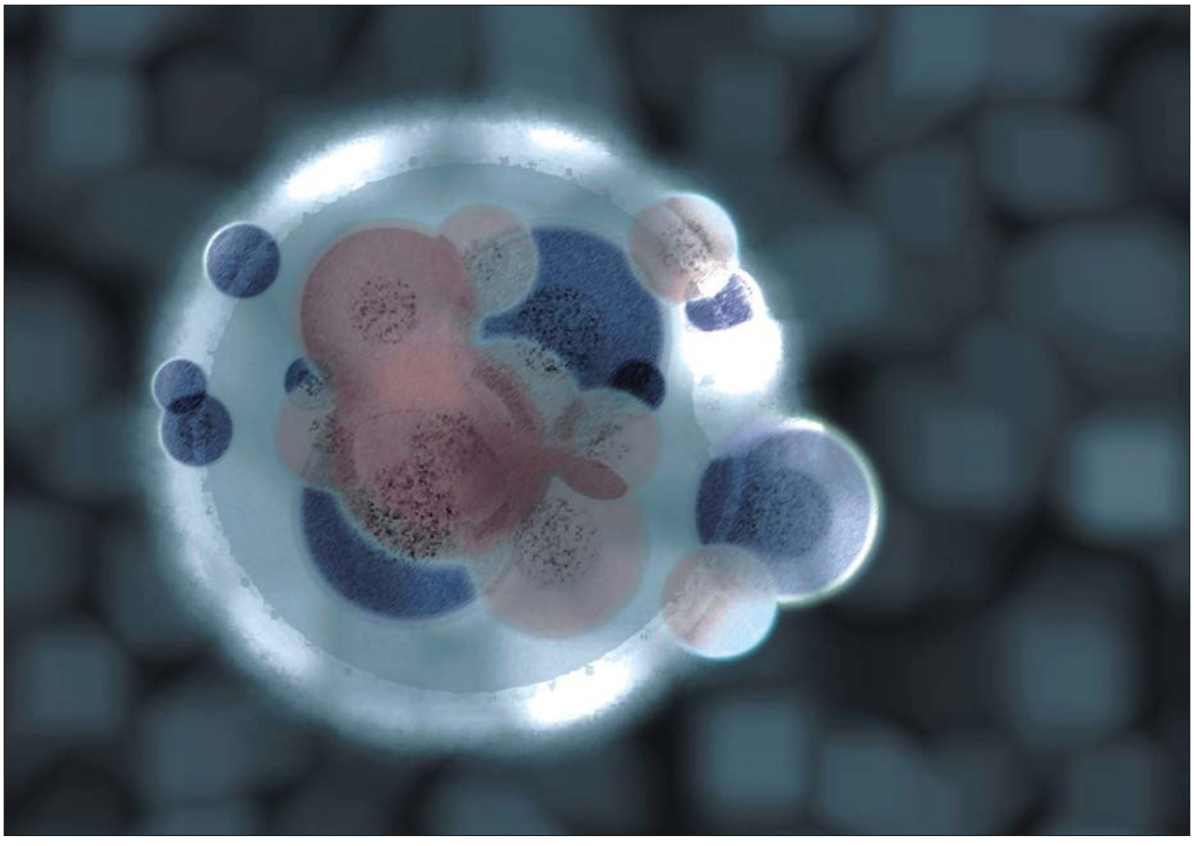

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The Role of Cell Wall Associated Peroxidase Activity and Xylem Sap pH in Limiting Leaf Expansion and Transpiration of Pepper *Capsicum annuum* L. Under Water Deficit

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Abstract: The effect of drying soil upon spatial distribution of leaf expansion, cell wall associated peroxidase activity and xylem sap pH in currently expanding leaves of *Capsicum annuum* L. was investigated. Soil water deficit reduced leaf expansion dramatically compared to plants which were well watered. Assayed cell wall associated peroxidase activity and xylem sap pH of droughted plants increased in the leaves to 10 fold than those found in well-watered plants of well-watered treatment. In the leaves of droughted plants highest cell wall associated peroxidase activities were observed in the regions of the leaf where expansion was occurring most rapidly in the well-watered plants. This increase in cell wall associated peroxidase activity and its spatial variation provides preliminary evidence that cell wall associated peroxidase activity may restrict cell expansion and subsequently leaf expansion in dicotlydenous species during periods of water deficit. Sap pH increased with increasing the intensity of water deficit and the differences were statistically significant as compared to those of well watered plants. Xylem sap of droughted plants was highest and well associated with lower transpiration rate.

Key words: Water deficit, *Capsicum annuum*, peroxidase activity, leaf expansion

INTRODUCTION

Leaf area is important for absorption of light energy to be used in photosynthesis, therefore leaf growth is one of the main determinants of plant production Monteith[1]. In pepper, a reduction in both total plant leaf area and leaf expansion rate due to water deficit effect, but exactly what are the causes of this reduction need to be explained. The belief that the reduction of leaf expansion rate is solely due to a reduction of leaf cell turgor during periods of soil drying based on the proposal[2] is now challenged by new evidence. Although a direct relation is often demonstrate between cell turgor and expansion rate. several investigators have shown the link may be questionable[3-5] and attention has therefore been directed to the chemical properties of cell wall and sap pH to investigate their roles in growth processes of growing tissues. Increase in xylem sap pH during soil drying and other stresses is frequently observed[6-9] which may give rise to increases in apoplastic pH of leaf tissues. The importance of pH in mediating physiological responses to stress has long been recognized and more recently it was acting as a stress signal that control gas exchange in leaves of plants growing in dry soil[5] .

Many studies are trying to explain the stomata response to different “abiotic stimuli”. Some evidence accumulating that stomata can respond to root or soil water status independent of reduction of leaf water potential[10]. ABA flowing from the root system via transpiration stream has been widely accepted to explain reduction in stomatal conductance under water limiting conditions. In this study, the effect of soil drying upon xylem sap pH and the spatial distribution of the cell wall peroxidase activity and leaf expansion rate of *Capsicum annuum* L was investigated to demonstrate the possibility that changes in cell wall-associated peroxidase activity and xylem pH could account for the observed reduction of leaf expansion rates during soil drying. The potential role of cell wall associated peroxidase activity and xylem sap pH in regulating leaf expansion and transpiration in *Capsicum annuum* L cv. Bell Boy growing in drying soil is examined by undertaking a spatial analysis of growth rate of currently growing leaf and comparing such a profile to that of cell wall associated peroxidase activity, extracted and assayed at the same spatial resolution.

MATERIALS AND METHODS

Experimental site: Seeds of pepper *Capsicum annuum* L. cv. Bellboy pepper were sown in small pots containing John Innes No. 2 commercial potting compost (Keith Singleton's Seaview Nurseries, Cumbria, UK). Seedlings

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were raised in a glasshouse with daily irrigation to drip point with tap water. After 14 days, seedlings of similar size and vigor were selected and transferred to large pots containing the same media of germination. Plants were raised in a controlled environment cabinet with a day/night temperature of 30/18°C, RH of 53/65%, under a photoperiod of 12 h and photosynthetic photon flux density of 400 μmol m^{-2} s^{-1} provided by eight 400 W tungsten halide lamps (Powerstar HQ1-T, Osram, Frankfurt, Germany). Plants were irrigated daily to the drip point with tap water until they recovered the transplanting shock. Half of the plants were given daily irrigation to drip point whilst the other half, water was withheld until plants show signs of early morning wilting.

Soil moisture content: Four pots were selected randomly from both well-watered and water deficit pots for the determination of soil moisture content. The samples were oven dried at 90°C for 72 h. and the moisture content of the soil sample was determined as g water/g soil.

Leaf water potential and sap collection for pH determination: A leaf was cut an immediately placed in a pressure chamber and pressurized until sap appeared at the cut end of the petiole. The water potential was recorded and the pressure was increased by a further 0.3 MPa. The first sap drop was blotted away[4]. The sap was then collected using a 2 ml syringe and place in a 1 ml appendorf tube and the pH was immediately recorded using a needle probe pH meter (Orion Needle pH electrode, Fisher Scientific U.K).

Spatial distribution of leaf expansion: Ten plants of similar size were selected for the measurements of daily growth increment in leaf length. The plants were arranged in growth cabinet into two experimental groups: control where plants were irrigated to drip point with tap water and stressed plants remained without irrigation for 10 days where plants were allowed to dry the soil. Two growing leaves were selected (leaves on node number 4 from the tip of the plant) from five control and stressed plants; one leaf was tagged and assigned for the measurement of daily increment in the length. The other leaf was divided into 10 equal sections by drawing grid lines 1.0 mm apart across the longitudinal axis of each leaf. The grid lines were made using ink and was given number based on leaf section. After 6 days from the onset of water withholding measurements of the length increment of each rectangle were recorded after 72 h. The same leaves were detached and dissected along the grid lines and the corresponding region of the two experimental groups was bulked to form one sample per analysis of

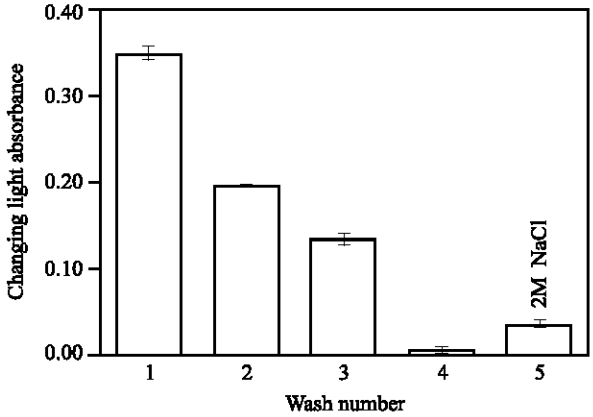

Fig. 1: Assessment of the ability of low and high ionic salt washes to remove and recover peroxidase activity from the leaf segments of Capsicum plants grown under wellwatered conditions. Values are means of 4 determinations. Bars represent standard error

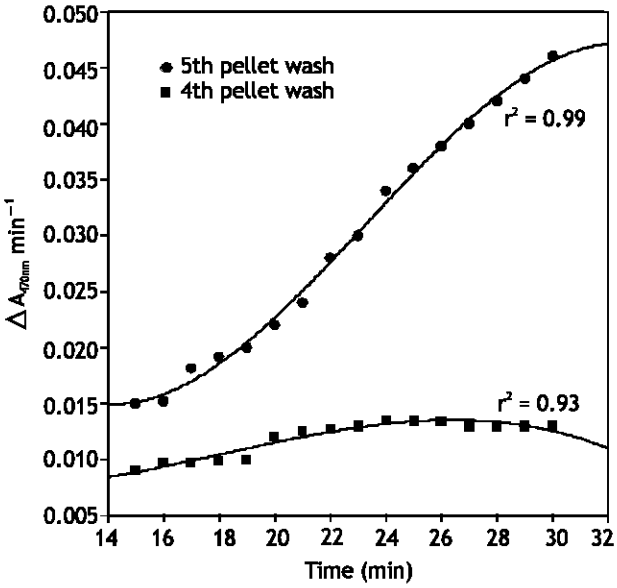

Fig. 2: Cell wall associated peroxidase activity plotted as change in light absorbance against time for the 4th and 5th pellet washes of well watered Capsicum plants

wall-associated peroxidase enzyme activity. Samples were collected in sealed microfuge tube held in ice. After collection the fresh weight of the bulked samples were determined and stored in freezer at -20°C for subsequent determination of cell wall peroxidase activity.

Validation of cell wall associated peroxidase extraction assay: A preliminary validity test of the peroxidase extraction assay was performed on leaf sample of well-

watered plants following the procedure described by Bacon *et al.*[11]. This test was run to determined suitable number of washes to be performed before using high salt concentration buffer to disrupt the association of activity within cell wall. The results of this test were shown in (Fig. 1 and 2). Cell wall associated peroxidase activity was found to be higher in pellet wash numbers five of well watered plant than the fourth pellet wash (low salt concentration). The first reading was taken after 15 min from the time when reaction was started showing peroxidase activity levels to be higher in the fifth pellet wash sample. Thirty minutes after the start of reaction peroxidase activity levels are three times greater in the fifth pellet wash compared to the forth wash. Absorbency increased over in a linear manner for both the forth and fifth wash. Peroxidase activity levels increased at an approximate constant rate with time in the fifth wash sample showing no sign of slowing the rate of activity with time (Fig. 2). However rates of activity with time slowed considerably after 26 min in the forth-wash sample with activity levels plateauing between 27 and 30 min.

Extraction and assay of cell wall peroxidase activity: Peroxidase extraction and assay were carried out as detailed by Bacon *et al.*[11]. The samples were homogenized with quartz sand (Sigma Poole, Dorset, UK) in ice cold buffer (50 mM sodium succinate, 10 mM calcium chloride, 1 mM dithiothreitol at a ratio of 10:1 buffer sample fresh weight. After samples had been ground, they were centrifuged at 2000 g for 5 min. The pellet was washed again in the same volume of 50 mM sodium succinate (Sigma Poole, Dorset, UK) to remove cytoplasmic peroxidase activity, before being re-suspended in an equal volume of the final extraction buffer containing 50 mM sodium succinate and 1 M sodium chloride to distrupt the association of activity with the cell wall. Each of the three low ionic concentration buffer washing stages were determined to reduce activity derived from tissue by c. 90% at all points in the elongation zone. The final high salt concentration was recovered contained c. 2 fold more activity than the final wash of the three low salt concentration washes. Activity was determined by assaying a 100 ul sample of the supernatant (equivalent to 10 mg fresh weight) using the guiaicol test. This 100 ul sample was added to 1 ml of 20 mM sodium phosphate buffer, which contained 276 ul guaiacol per 50 ml of buffer.The reaction was started by adding 200 ul of 0.03% hydrogen peroxide in distilled water (w/w). The concentration of hydrogen peroxide (the H donor) and guaiacol (the H acceptor) used, gave a linear change in absorbancy over 20+ min. The reaction was mixed and incubated at 25°C in 1.5 ml spectrophotometric cuvettes. The absorbancy of the solutions at 470 nm was then measured after 20 min using Cecil series 2 spectrophotometer (Cecil Instrument, Cambridge, UK). All buffer, extraction and assay solutions were corrected to pH 5.5, a presumed pH of the expanding plant cell wall under well watered conditions. The assay of extracted activity at different pH values shows similar trend as shown by Bacon *et al.*[11]. The cell wall peroxidase activity is measured based on this reading of the absorbance on the spectrophotometer.

RESULTS

Soil moisture content: Figure 3 shows the moisture content of the growth medium of well-watered and stress plants. In the well-watered treatments, the moisture content remained high throughout the experimental period, this suggests that the plants did not suffer any water deficit stress. The moisture content where water was withheld was decreased progressively with days of withholding water. From day 5 onwards, the moisture content of water deficit treatment was significantly lower than that in well-watered treatment indicating that the plants were undergoing water deficit stress and wilting signs appeared in the plants with the drooping of the lower most leaves.

Midday leaf water potential: Midday leaf water potential of well-watered plants remained low throughout the experimental period (Fig. 4). However the midday leaf water potential of water deficit plants remained similar to that of well-watered plants until day 5 after which the midday leaf water potential decreased as the duration of water withholding increased. This shows that by day 6 of withholding water, the midday leaf water potential of water deficit plants had fallen to about -0.7 MPa significantly lower than that of well-watered plants. The lowest value of midday leaf water potential was observed 10 days after water withholding, where the midday leaf water potential of water deficit treatment plants follow similar trend of the media moisture content. The present results clearly demonstrate that the midday leaf water potential of the plants is largely influenced by soil water deficit.

Xylem sap pH: Figure 5 shows the values of the xylem sap pH of the plants growing under well-watered and water deficit conditions. The xylem sap pH of well-watered plants remained relatively low compared to droughted plants throughout the experimental period. However the pH of xylem sap of water deficit plants increased as duration of water withholding increased. By day 5 after

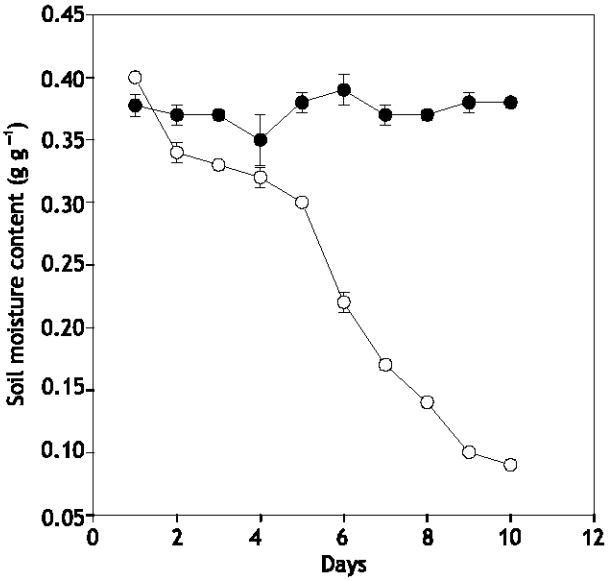

Fig. 3: Moisture content of plant growing media which was either well-watered (closed symbols) or allowed to dry over 10 days (opened symbols). Each point is a mean of 4 determinations. Bars represent standard error

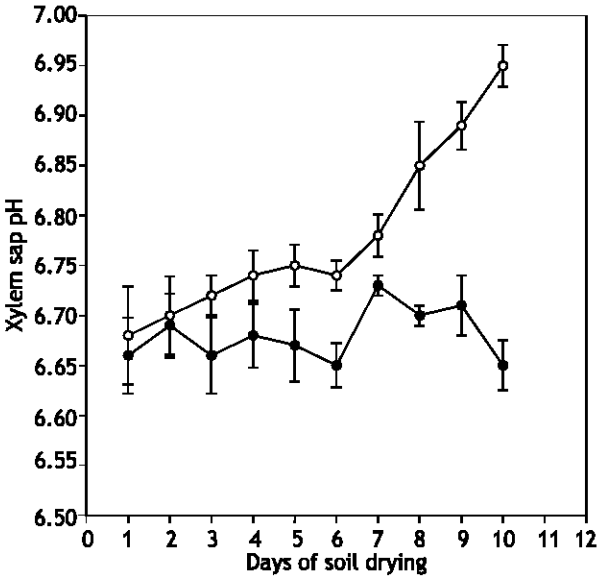

Fig. 5: Effect of soil drying on xylem sap pH of well-watered Capsicum plants (closed symbols) and stressed plants that grown in soil allowed to dry over 10 days (opened symbols). Each point is of 4 determinations. Bars represent standard error

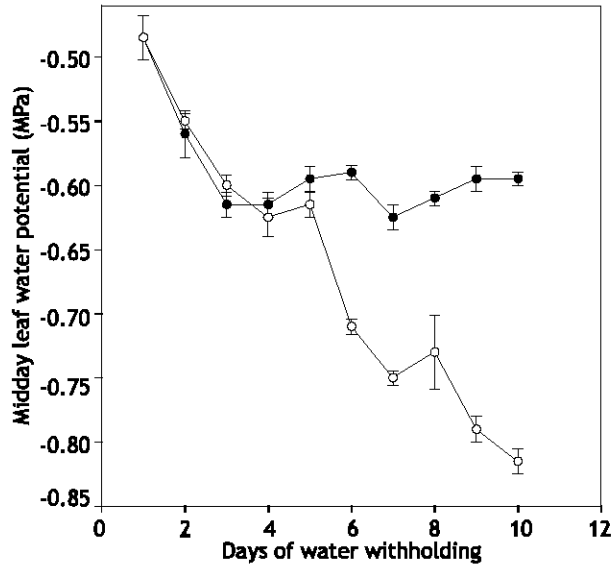

Fig. 4: Effect of soil drying on midday leaf water potential of well-watered plants (closed symbols) and droughted plants where soil was allowed to dry over 10 days (opened symbols). Each point is a mean of 4 determinations. Bars represent standard error

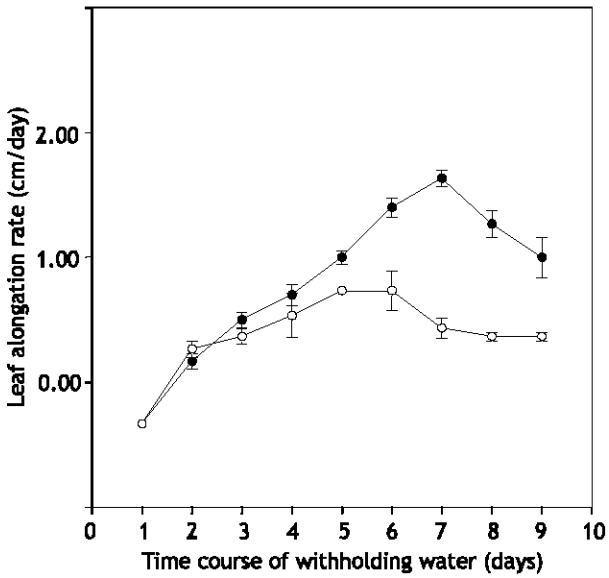

Fig. 6: Leaf expansion rate of well-watered Capsicum plants (closed symbols) and water deficit plants where water withheld for 10 days (opened symbols). Each point is a mean of 4 replicates. Bars represent standard error

water was withheld, the xylem sap pH of water deficit treatments were significantly higher than that of the well-watered plants.

Leaf expansion rate: Leaf expansion rate of both well-watered and water deficit plants is presented in Table 1 and Fig. 6. Leaf expansion rates of well-watered treatment plants increased at higher rates as compared to that of

Table 1: Leaf elongation rate (cm day^{-1}) of currently expanding leaf of well-watered plants and plants growing in drying soil for 9 days

Days from imposition of treatments	Control plants	Droughted plants
1	0.00	0.00
2	0.375±0.096	0.450±0.100
3	0.625±0.957	0.525±0.096
4	0.775±0.126	0.650±0.265
5	1.000±0.082	0.800±0.000**
6	1.300±0.115	0.800±0.000**
7	1.475±0.096	0.575±0.126**
8	1.200±0.163	0.525±0.050**
9	1.000±0.245	0.525±0.050**

Significance of differences were tested using a student t-test (Sigma Stat for windows version2, Jandel Scientific, Erkarth, Germany) ** indicates significance at the 1% level

Table 2: Peroxidase activity (arbitrary units) of leaf number 4 from the top of well-watered plants and plants growing in soil drying for 9 days

Leaf region	Control plants	Stressed plants
1	0.029±0.010	0.262±0.015**
2	0.024±0.009	0.224±0.011**
3	0.023±0.003	0.198±0.008**
4	0.020±0.001	0.190±0.009**
5	0.016±0.001	0.171±0.013**
6	0.014±0.001	0.118±0.013**
7	0.011±0.002	0.108±0.013**
8	0.014±0.005	0.108±0.007**
9	0.012±0.004	0.072±0.020**
10	0.012±0.004	0.068±0.020**

Data were means of 4 determinations± standard error. Significant differences were tested using a student t-test (Sigma Stat. for Windows version 2, Jandel Scientific, Erkrath, Germany). ** Indicates significance at the 1% level

water deficit. A significant decrease in leaf elongation rate of currently expanding leaf caused by soil drying treatment was first detected 3 days after withholding water, when rates within control plants were still maximal. However, the leaf expansion rates of the well-watered plants slowed on day 8 and 9 indicating that termination of leaf elongation.

Spatial distribution of leaf expansion: In this study the increment of leaf expansion rate along each sector in both well-watered and droughted plants was recorded at 7 days after imposition of treatments and subsequent 72 h period is presented in Fig. 7. Leaf expansion rates reached maximal rate approximately 1.4 mm in sector1, 2 and 3 at the base of the leaf of well-watered plants. The general trend of decrease in both well watered and a water deficit plant is similar from the base to the tip of the leaf. This shows that leaf expansion rate of water deficit plants declined successively with distance from the base to the tip of the leaf. This decline in leaf expansion was obvious with distance from the leaf base with the greatest rates of decrease of leaf expansion with distance occurring within the first 25 mm of leaf measured from the base compared to that of well-watered control plants.

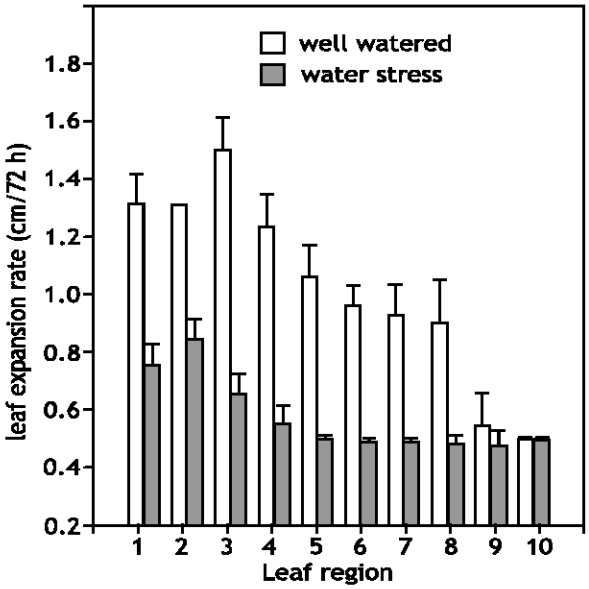

Fig. 7: Spatial distribution of leaf expansion rate of well watered Capsicum plants (and water deficit plants where water withheld for 10 days (shaded). Each point is a mean of 4 replicates. Bars represent standard error

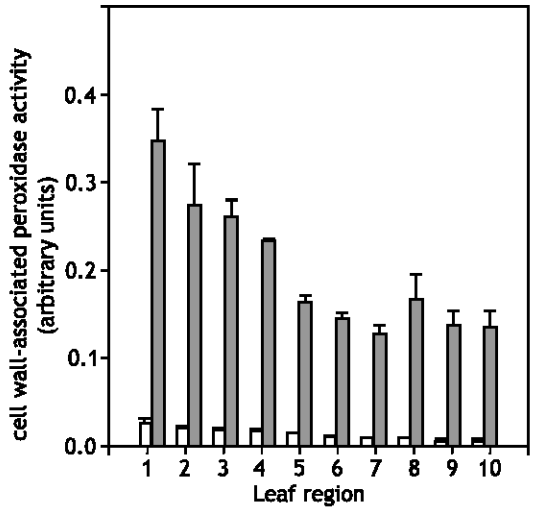

Fig. 8: Spatial distribution of cell wall associated peroxidase activity of well- watered Capsicum plants (not shaded) and water deficit plants where water was withheld for 10 days (///-shaded bar). Each point is a mean of three determinations. Bars represent standard error

Spatial distribution of cell wall-associated peroxidase activity: The spatial variation in cell wall associated peroxidase activity in the leaves of well-watered and droughted treatment plants is shown in Fig. 7. Cell wall associated peroxidase activity levels in the leaves of

droughted plants are significantly higher compared to that observed in the leaves of well watered plants (Table 2). Levels of peroxidase activity were about 10 times higher in the base of the water deficit plants.

Relationship between spatial distribution of peroxidase activity and leaf growth: Figure 8 shows the relationship between spatial distribution of peroxidase activity and leaf expansion rate of Bellboy pepper plants. The maintenance of growth in the very basal region of the leaf growing in both well-watered and drying soil corresponded with the basal position in the elongation zone where peroxidase activity was seen to be increased. In well-watered plants although activities were far lower than those recorded in drying soil but leaf expansion maintained high rates as compared to that of drying soil. There are good correlation between increased peroxidase activity level and reduction of leaf expansion rate in the basal part where elongation takes place of the both droughted plants and well watered plants.

DISCUSSION

When the soil had been allowed to dry for a total of 9 days, leaf elongation rate (LER) was markedly reduced compared to the leaf from well watered plants. In well watered plants, LER reached maximal rates of around 1.3 to 1.48 cm day, c. 6-7 days in the experimental period. A significant decrease in LER caused by the soil drying treatment was detected after 4 days after withholding water (Table 1). The results showed a clear indication of the absence of linearity between plant water relations and leaf growth which agreed with many other reports on crop species[12-15].The mechanistic processes underline the control of leaf expansion of pepper leaves may involves cell wall enzymes, ABA and changes in ionic composition. Ismail *et al.*[12] did not detected the presence of xylem sap ABA in droughted pepper plants that may have a greater influence to initiate early reduction in leaf expansion in pepper culivars. However, the role of ABA mediating leaf expansion should not be ruled out totally. Although, the significance of accumulations of ABA in influencing leaf expansion has been questioned[16,17] there may be other role concerning the active site of ABA dependent growth restriction. The present study, however, did not elucidate how the ability of ABA to regulate enzymes proposed to catalyse the control of leaf expansion which may include cell wall activation of peroxidase synthesis.

In this study significant higher levels of cell wall-associated peroxidase activity occurred after 6 days of drought treatment. The maximal increases occurring in areas of the leaf known to be expanding most rapidly in the watered control plants. Maximal rates of decrease in leaf expansion between well watered and water deficit plants were in the basal region of the leaf. This phenomenon of greatest reduction in leaf expansion rate in the basal regions of droughted plants was also observed in four cultivars of *Helanthus annus* L.[18]. These increased levels of activity correlated with the much reduced growth rate in droughted plant leaves suggesting that cell wall associated activity levels may act to control leaf expansion during drought in pepper plants. The changes in peroxidase activity associated with growth of pine hypocotyls are that of apoplastic peroxidases[19]. These finding are further supported on Cicer arietinum epicotyls who found that the specific activity of cell wall associated peroxidase termed PX-2 increases with epicotyl age forming an inverse relationship with their growth capacity[20]. Evidence suggests cytoplasmic peroxidase activity levels are unaffected by drought treatment. There was no significant rise of cytoplasmic peroxidase activity following an 8-day drought episode[21]. Similarly, no rise in ascorbate peroxidase activity in the bundle sheath cells of maize following a severe drought treatment, where water was withheld for 18 days[22]. The responses of cell wall associated peroxidase activity levels in *Lolium temulentum* L. to drying soil, were found to be 200-300% higher in the leaf base of plants subjected to a 9 day drought treatment, compared to well-watered control. Activity was found to return to levels close to those in the control plants outside the zone of leaf expansion[11]. Although a direct comparison on peroxidase levels between monocotyledonous species reported above and in Capsicum plants is speculative due to differing mechanism of leaf expansion. However, there was report suggesting peroxidase had directly involved in growth of dicots as reported in peanut (*Arachis hypogaea* L.) hypocotyls[23]. Peroxidases has directly regulate cell elongation through catalizing the crosslinks of wall phenol compound[23]. There was a significant increased in cell wall associated peroxidase activity in the leaf elongation zone and subsequent decrease in growth rate of *Capsicum annuum* L. leaves. The leaves of droughted plants are not as long as those of the control plants. The increase in cell wall associated peroxidase activity was greater in droughted plants than in the controls, probably because the higher cell wall peroxidase activity could increase the amount of cross-linking within the cell wall and prevent further cell expansion and leaf growth in drought conditions and would explain the decrease in leaf growth as peroxidase levels increased. Similar results were reported in previous experiments in grasses[24,25].

When a plant is stressed and the leaves wilt it implies that the mesophyll cells within a leaf have lost turgor.

Thus a loss of turgor by mesophyll cells implies that the osmolality of the leaf apoplast is higher than the cytoplasm of those mesophyll cells. Thus, tension in the xylem, therefore, implies a cycle of signaling events in the xylem parenchyma cells that proceeds up the vascular tissue leading to increase pH[6,9]. Similarly the results of this experiment showed that the pH of the xylem sap of water deficit plants increased. Wilkinson *et al.*[9] suggested that the rise in xylem sap pH could account for the closure of stomatal aperture by accumulation of ABA in the apoplast adjacent to the stomatal guard cells. This pH increase was suggested to function as an early signal of reduced soil water content to the leaf, mediated by ABA[14]. The interactive effects of cell wall pH and enzymes such as cell wall associated peroxidase mediating leaf growth has also been reported in many other studies[26,27]. It would be suggested from the present study that the effect of sap pH might cause an accumulation of ABA to concentrations that induce reduction of expanding leaf growth rates. The increase of sap pH (alkaline) may account for the accumulation of ABA, which in turn can affect the growth rates of expanding tissues and stomatal closure.

These findings show that leaf growth of pepper plants is sensitive to changes in soil water deficit. Early leaf growth inhibition was brought by the presence of non-hydraulic signals from the roots. The increased in cell wall associated peroxidase activity and pH may play a major role in mediating leaf expansion of pepper plants exposed to drought conditions. These activities coupled with ABA and ethylenes are increasing implicated in coordinating the responses and shoots to change in soil water availability. The interactive role merit further investigation in pepper plants to explore the fundamentals in those factors that may govern plant responses for crop improvement programme in the tropics and semi-arid regions.

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