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PROTEOMIC ANALYSIS AND PEROXIDASE ACTIVITY IN *Aster tripolium* UNDER OXIDATIVE STRESS IN AMBIENT AND ELEVATED CO_2

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ABSTRACT

The combined effects of NaCl-salinity and elevated CO_2 on the polypeptide levels in leaves of *Aster tripolium* grown under ambient (ca. 370 ppm) and elevated (520 ppm) CO_2 in different concentrations of saline irrigated water were examined by two-dimensional polyacrylamide gel electrophoresis. Silver nitrate stain gels were analyzed to identify changes that resulted when plant grown in the presence of 375 mM NaCl at ambient CO_2 level. The protein patterns for control and salt-stressed seedlings were qualitatively changed. This observation was mainly noticeable in young leaves which was not exposed to salinity stress. It is shown more polypeptide proteins through wide range of pH. In contrary, leaves exposed to salinity stress revealed narrow polypeptide pH range proteins. Also, the control old leaves which was not exposed to salt stress showed limited pH range and lower polypeptide numbers compared to young leaves.

The effect of NaCl on peroxidase activity under 0,125,250, 375 and 500 mM NaCl was studied at the ambient CO_2 and elevated CO_2 levels. Peroxidase activity increased under NaCl salinity and the degree of elevation in the activity was salt concentration dependent. Nevertheless, a great activity was recorded in stressed leaves comparison to control leaves. Furthermore, peroxidase activity was changed to lower activity in high level of CO_2 corresponding to ambient level of CO_2 The study revealed that *Aster tripolium* showed distinct differences in peroxidase activity behaviour under different environmental condition. The correlation coefficient between peroxidase activity under different salinity stress and high level of CO_2 was strongly negative correlation, which means that plant will not reveal stress response of peroxidase activity under elevated CO_2. Lowering of peroxidase activity under both salinity stress and higher level of CO_2 indicate to the important and unique role of halophyte i.e. *Aster tripolium* in this study for removing, cleaning and remedy environmental air from higher level of CO_2 and decrease the green house effect.

Keywords: *Aster tripolium,* Oxidative and salt stress, Peroxidase, 2-D-Electrophoresis. CO_2 and Green house effect.

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INTRODUCTION

Numerous studies have investigated the adverse effects of salinity on antioxidant enzyme activities. In addition, it has been demonstrated that salt treatment of cotton plants resulted in an increase in peroxidase and glutathione reductase (GR) activities in the more sensitive cultivar (Gossett *et al.,*1994*).* Moreover, an increase in the activities of GR and ascorbate peroxidase (Apx) was also detected under the influence of salt stress in Pea and Maize (Mittova and Igamberdiev (1998). The effect of salt stress on the pattern of protein synthesis in leaves of different graminaceous taxa under salt stress by SDS-PAGE was used to identify polypeptides whose synthesis was altered and whose are new expressed were mentioned by Ali, *et al. (*2006*).*

Influence of NaCl salinity on the oxidative stress of growing plants and more specially the ability of the tissues to generate reducing power under stressful environment of salinity remain to be understood. Besides, seedling stage is one of the most critical stages for salt damage during the life cycle of plants. The behaviour and reaction effect of some enzymes and salt stress was studied by Ritambhara *et al.* (2000). An increase in the peroxidase activity is a common response to oxidative and abiotic stress (Olmos *et al.,*1997*)* and (Fieldes and Gerhardt, 1998).

This work was concerned with peroxidase activity in order to understand the role of peroxidase in conferring stress resistance. Also, the present work will undertaken the reflection effect of peroxidase in *Aster tripolium* grown under different salt concentrations in the environmental condition and in elevated CO_2. Also, proteomic analysis for control and treated samples of *Aster tripolium* under salt stress comparison to young seedling leaf control was also studied.

MATERIALS AND METHODS

Design and Treatments

This experiment was carried out in 2001 in Justus-Liebig-University Giessen, Institute of Plant Ecology, Giessen, Germany. Plants were irrigated with five different salinity levels, tap water, 125, 250, 375 and 500 mM NaCl, in a quick"=check-system in open"=top chambers under ambient (ca. 370 ppm) and elevated (520 ppm) CO_2. The effects of the major constraints of salinity on plant enzyme activity of peroxidase and proteomic analysis of young, old control and treated representative leaves with 375 mM NaCl at ambient (ca 370 ppm) CO_2 were studied.

Enzyme extraction and assay (Peroxidase)

Enzyme extraction

One gram of fresh plant leaf tissues in 3 ml of 0.1 M phosphate buffer pH 7 homogenized and grinding with a pre-cooled mortar and pestle. The homogenate was centrifuged at 18000 g at 5 ^oC for 15 min. The resultant supernatant was stored on ice till the assay is carried out and used as enzyme source within 2-4 h. for assaying peroxidase activity.

Assay of peroxidase activity

Total peroxidase activity in the extracts was assayed as described by Sadasivam and Manickam (1992). In a cuvette put the reaction mixture which consisted of 3 ml buffer phosphate (0.1 M pH 7), 0.05 ml guaiacol solution (0.24 mg/100 ml distilled water), 0.1 ml of crude enzyme extract and 0.03 ml hydrogen peroxide solution (0.14 ml of 30% H_2O_2 make up to 100 ml with distilled water) was added and mixed well to initiate the reaction which was measured spectrophotometrically at 436 nm. The enzyme activity units/liter = 500/(time required in minutes to increase the absorbance by 0.1).

Protein determination

Protein in the extracts were quantified by the method of Bradford (1976) using bovine serum albumin as the standard.

Proteomic analysis.

Protein extraction from *A.tripolium* leaves for 2-D-SDS-electrophoresis

0.1 gram leaves with 0.1 g poly vinyl pyrolidine (PVP) was mortared using pestle in liquid nitrogen to fine powder. Homogenate washed with 1.5 ml solution (1% TCA in acetone) the previous 1.5 ml containing 75 ul dithiotheritol (DTT) added before wash from 1 M DTT. Mixture let to precipitate for 1 h. at -20 ^{o}C. After that, samples were centrifuged (12000 rpm) at 4^{o}C for 15 min. Pellet resuspended and rewashed again with 1.5 ml (1%TCA/Acetone) included 75 ul DTT. The previous step made more once till the sample pellet appear white. After that, pellet rehomogenized in 1 ml ice-cold ethanol contain 50 ul DTT (50 mM), DTT added freshly from stock solution. Let samples to precipitate for 45 min. at -20 ^{o}C and centrifuge (12000 rpm) for 10 min. at 4^{o}C. Rehomogenized pellet again in 1 ml cold ethanol/DTT, then centrifuge 10 min., 4^{o}C. Pellets were stored overnight at -20 ^{o}C. Then, pellet was homogenized in 1.5 ml lysis-buffer. Next, samples were shaken in water bath for 2 hours adjusted at 33^{o}C, then centrifuge for 30 min. at 4^{o}C. The supernatant containing extracted leaves protein used for 2-D SDS-PAGE separation. All centrifuge steps performed at 4^{o}C and 12000 rpm.

Protein Separation

Protein samples were stored at -20 ^{o}C. Analytical 2-D PAGE was carried out in proteomic instrumental system. The first-dimensional isoelectric focusing (IEF) was done according to O'Farrel (1975) and Mayer *et al*. (1987) with modification describeed by Ouelhazi *et al*. (1993). The gels contained 3-10 carrier ampholytes and were loaded with 100 ug proteins on 12 cm IEF rod gels (1.5 mm diameter) and rehydrated (20 hour) at 20 ^{o}C (15 uA/strip), then gradient up to 3500 volt (8 hours), at the end hold at 3500 volt (14 hour). SDS-PAGE was performed under constant current intensity (15 uA/gel). Molecular weight markers ranging from 14.4 to 90 kDa (Pharmacia) were co-electrophoresed to estimate molecular weight of polypeptide chains. After running, the gel was stained with silver nitrate as described by Heukeshoven and Dernick (1985).

Statistical analysis.

Statistical analysis was performed according to Satgraphics Plus ver 7 (1993).

RRESULTS AND DISSCUSSION

Peroxidase activity at Ambient CO_2

Data illustrated in Fig.1 showed peroxidase activity of *A.tripolium* plants irrigated with different concentrations of saline water and grown under ambient level of CO_2 (370 ppm). Total peroxidase activity of the crude enzyme extracts revealed that enzyme activity was increased starting from control to reach maximum activity at 125 mM NaCl salinity. However, under salinity stress of NaCl the enzyme activity is still high at 250 mM NaCl,

meanwhile the raising NaCl concentration resulted in lowering in peroxidase activity to be close to control level. On the other hand, raising NaCl concentration return peroxidase activity to elevate another again, but not much. These results mean that peroxidase activity was a salt-dependent responsive enzyme. Also, showed that peroxidase activity was the best reflected parameter to salinity degree.

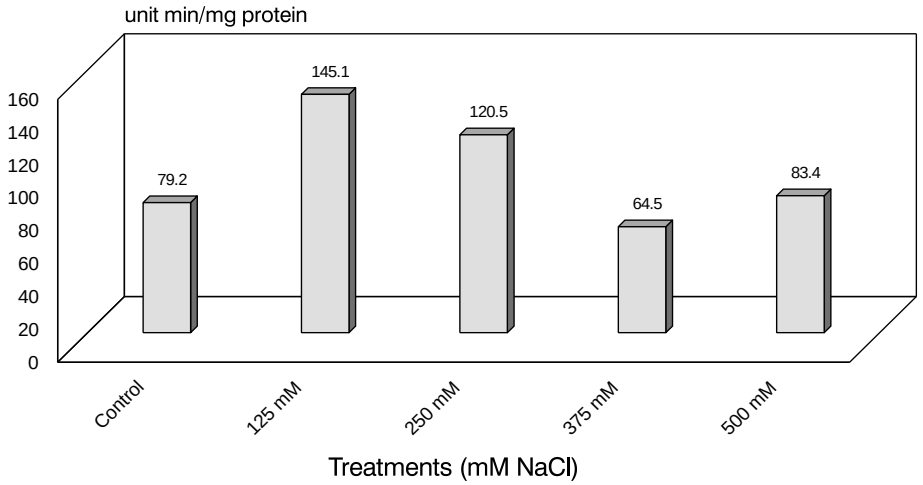

Fig.1. Peroxidase activity in *Aster tripolium* irrigated with different concentrations of saline water (mM NaCl) under ambient level of carbon dioxide (370 ppm).

Peroxidase activity at Elevated CO_2

Data illustrated in Fig.2 showed peroxidase activity of *A.tripolium* plants irrigated with different concentrations of saline water and grown under elevated level of CO_2 (520 ppm). Data revealed that peroxidase activity showed maximum value at 375 mM NaCl salinity. Meanwhile, lower peroxidase activity values were shown at 125, 250 and 500 mM NaCl salinity. Alteration of peroxidase activity under this conditions revealed two things: Firstly, peroxidase activity was responsible parameter to salinity degree as previously found in ambient CO_2 level. Secondly, the lowering in peroxidase activity reflect the ability of *A.tripolium* to utilize and consume the environmental CO_2 without increasing its activity, which mean that plant was not under salinity stress. On another word, plant in salinity stress with high level of CO_2 could adapted to this stress and still grow without raising its activity. Which pointed to suitability and ability of this plant to grow in environment polluted with higher level of CO_2 . In addition that plant could have a defense mechanism to protect itself from enhanced production of oxygen free radicals which responsible for peroxidation of membrane lipids and the degree of peroxidative damage of cells was controlled by the potency of antioxidative peroxidase enzyme system.

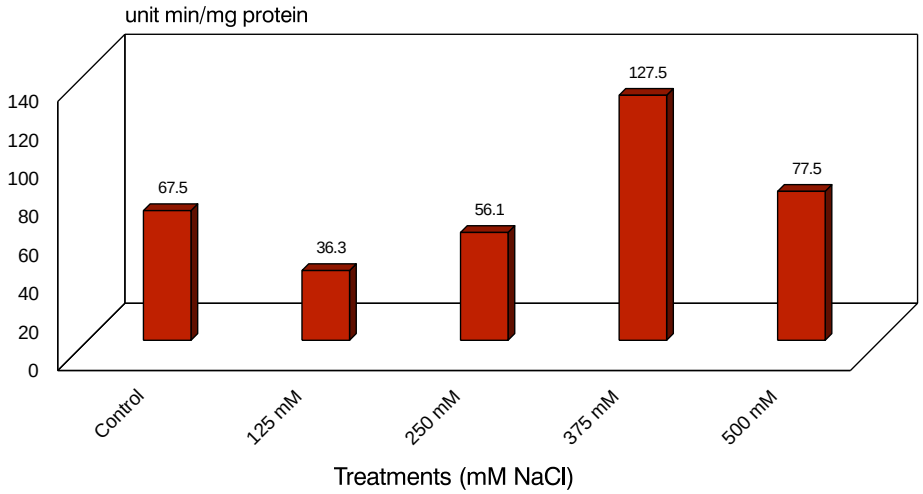

Fig.2. Peroxidase activity of *Aster tripolium* irrigated with different concentrations of saline water (mM NaCl) under elevated level of carbon dioxide (520 ppm).

These results are in harmony with Siegel, (1993) and Sancho *et al.* (1996) who reported that total peroxidase activity was increased in response to salinity. Also, Sancho *et al*. (1996) stated that the increase of total peroxidase activity in the medium of the salt adapted cells reflect the changed mechanical properties of the cell wall, which in turn could be related to the salt adaptation process since cell wall properties are known to be modified by salt stress and earlier reports (Bradly *et al.,* 1992*;* Chen *et al.,* 1993) Link total peroxidase activity to change in cell wall and cell membrane integrity properties under salt stress. Also, these results are in accordance with Sreenivasulu *et al.* (1999) who reported that exposure to salinity resulted in changes in the induction of total peroxidase activity and its isozymes and such alterations in the induction and its isoform patterns vary between cultivars. Nevertheless, they also added that relatively tolerant nature of cultivar could be due to induction of specific peroxidase isozyme and the cultivars differed in their ability to respond to salinity by triggering these peroxidase gene expression. Moreover, according to Eshdat *et al. (1997)* peroxidases are a family of multiple isozymes that catalyze the reduction of H_2O_2, and thus help to protect the cells against oxidative damage. This result can supply information on the possible involvement of activated oxygen species in the mechanism of damage by NaCl stress, and also could allow deeper insight into the molecular mechanisms of salt tolerance to salt induced oxidative stress. To better understand the changes caused by salt stress.

Peroxidase activity Behaviour under ambient and elevated CO_2

Data depicted in Fig.3 revealed the inversion behaviour of peroxidase enzyme under ambient and elevated CO_2. It has been pointed out that peroxidase behave stress behaviour under ambient condition during oxidative stress from salinity. Meanwhile, it behaves completely opposite behaviour

under elevated CO_2 during oxidative stress of salinity. This opposite behaviour mean that elevated CO_2 reduce oxidative stress for this plant under salinity stress. This remarkable note could be confirmed from its activity which increased at high level of NaCl 375 mM, which mean at this point the plant begin to give its response for oxidative stress. This opposite trend for Aster under this condition should be taken with more consideration. This results are in harmony with Heath and Packer (1968) they reported that peroxidase isozymes play a key role in salt tolerance. Also, Sreenivasulu *et al*.(1999) stated that the degree of increase peroxidase activity was found to be dependent on severity and duration of stress.

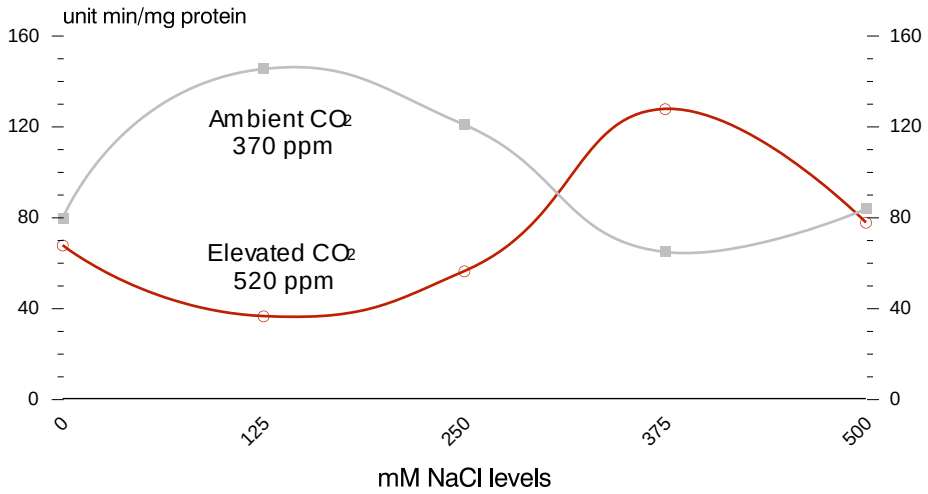

Fig.3. Behaviour of Peroxidase Enzyme Activity in *Aster tripolium* under Ambient and Elevated CO_2. (370 & 520 ppm) irrigated with different levels of saline water.

Proteomic analysis

Proteomic analysis for young leaf of *A.tripolium* compared to old and treated plant under 375 mM NaCl salinity stress at ambient CO_2 was depicted in Figs. 4,5,6. The analysis were limited to the polypeptide changes that were easily visible. Proteomic analysis revealed that polypeptide chains of young leaf were numerous and dispersed in wide range of pH with different molecular weight ranged from 94 to lower 14.4 as shown in Fig. 4. This mean that plant did not suffer from any kind of stress and was in a normal condition. Further, from 2-D-electrophoresis it was not accumulated in limited zone.

Stained gels (Figs.4-6) revealed that the polypeptide patterns were strikingly similar between control old leaf and salinity samples. Treatment by 375 mM NaCl results in an increase in the net synthesis of some proteins and a decrease in the synthesis of others as shown from arrow in Fig (4,5 & 6). Also, it is obviously shown a new peptide in stressed samples and a decrease in the pattern number in stressed sample comparison to non stressed. In addition a concomitant induction of unique “stress proteins” was observed in stressed sample (Fig.6). The most striking change in leaf protein of salt-

grown plants is a significant increase in the net synthesis of polypeptide wit *pI* approximately 5. The decrease in polypeptide was noticeable in both control old and stressed plant leaves. New polypeptide is considered to be a salt-tolerant plant against salinity stress. The increase in the polypeptide during salt stress could be important in the adaptation of the plant to the saline conditions. This results are in harmony with Majoul *et al.* (2000). Furthermore, Baiar and Dietz (1996) stated that peroxiredoxins are a new group of peroxide scavenging enzymes sharing no homology with any other isoperoxidase known so far.

Regarding to Figs. 4 and 5 it was found that, proteomic analysis for old and stressed leaf of *A.Tripolium* was accumulated in both narrow zone and range of pH mainly acidic range between 4 and 7 this range was reduced to 4-5 in stressed plant. This result means that, firstly, plant synthesis protein was differed according to the type of stressed, old leaf is suffering from senses stress and the other leaf suffer from salinity stress. Secondly, the type of synthesis protein under salinity stress was differed, it tend to be acidic in its isoelectric point. This finding are in agreement with Ali and Eisa (2001) they found that under salinity stress the plant tend to decrease the pH to acidic pH. Furthermore, Heath and Packer (1999) reported that peroxidase is an important defence system of plants against oxygen free radicals. Nevertheless, but the degree of elevation in peroxidase activity was to be dependent on severity and duration stress. Not only that, but they also found a greater activity of acidic peroxidases in 5-day old seedling of tolerant variety under NaCl stress could be related to the salt adaptation of this variety.

Fig.4. Patterns of silver nitrate stained proteins extracted from leaves of *A.tripolium* control young leaves without salinity stress under ambient level of CO_2

Fig.5. Patterns of silver nitrate stained proteins extracted from leaves of *A.tripolium* and old leaves without salinity stress under ambient level of CO_2

Fig.6. Patterns of silver nitrate stained proteins extracted from leaves of *A.tripolium* leaves with salinity stress (375 mM NaCl) under ambient level of CO_2

In addition this acidic peroxidases might be involved in cell membrane integrity, regulation of early seedling growth under salt stress conditions as demonstrated in some plant species (Gaspar *et al.,* 1991). This finding was confirmed in the present work, on other word acidic peroxidase this enzyme give the optimum activity at acidic pH which confirm the current finding in the present study.

Conclusion

The effect of salt stress on the pattern of protein synthesis in leaves of *Aster tripolium* by 2-D-PAGE was used to identify polypeptides whose synthesis was altered or new expressed . The present study showed high total peroxidase activity at ambient level of CO_2 and opposite trend like the mirror image of peroxidase activity was shown in elevated level of CO_2 in *Aster tripolium* grown under different levels of saline conditions. The study revealed that *A. tripolium* showed distinct differences in peroxidase activity behaviour under different environmental condition. The results show that *A. tripolium* is a promising cash crop halophyte. Halophyte allows the use of saline irrigation water and the reclamation of saline soils, and its sustainable use can help feeding the growing world population. Additionally, not only Aster will clearly benefit from rising CO_2-concentrations in future, but also it can counter global climate change by sequestering CO_2.

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التحليل البروتينى ثنائى الإتجاه ونشاط البيرأكسيديز فى نبات أستر ترايبوليوم النامى فى مستويين من ثانى أكسيد الكربون تحت تأثير الإجهاد التأكسدى

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تهدف هذه الدراسة لمعرفة مدى تأثر النشاط الإنزيمى لإنزيم البيرأكسيديز وتأثير ذلك على البروتينات الببتيدية فى نبات أستر ترايبوليوم تحت تأثير درجات مختلفة من الإجهاد الملحى المسبب لدرجات متباينة من الإجهاد التأكسدى . لذلك تم اختيار نبات أستر ترايبوليوم وتم زراعته فى أصص بنظام "quick-check-system in open-top chambers" تحت تأثير خمسة تركيزات مختلفة من الملوحة كالتالى: كنترول ، ١٢٥ ، ٢٥٠ ، ٣٧٥ ، ٥٠٠ مليمولر من كلوريد الصوديوم وذلك تحت مستويين من تركيز ثانى أكسيد الكربون الجوى المستوى الطبيعى (370 ppm) ومستوى يعادل مرة ونصف المستوى الطبيعى (520 ppm) .

أظهرت النتائج تأثر النشاط الإنزيمى لإنزيم البيرأكسيديز بزيادة التركيز الملحى حتى ١٢٥ مليمولر ثم بدأ فى الانخفاض بزيادة التركيز الملحى حتى ٢٥٠مليمولر كلوريد صوديوم لكن بدرجة أعلا قليلا من الكنترول الغير معرض للإجهاد التأكسدى الملحى ، وبزيادة التركيز الملحى حتى ٣٧٥ مليمولر بدأ نشاط البيرأكسيديز فى الانخفاض ثم عاد للارتفاع مرة أخرى عند تركيز ملحى ٥٠٠ مليمولر NaCl . وفى المقابل أظهرت النتائج أن نشاط البيرأكسيديز فى وجود تركيز عالى من ثانى أكسيد الكربون قد حدث له نقص واضح عند التركيز الملحى المنخفض ثم عاد النشاط الإنزيمى للارتفاع مرة أخرى ليصل إلى درجة مقاربة للنشاط الإنزيمى للكنترول ثم يواصل الارتفاع حتى تركيز ٣٧٥ مليمولر كلوريد صوديوم ثم ينخفض مرة أخرى عند تركيز ٥٠٠ مليمولر من NaCl. كما أظهر التحليل الإحصائى وجود علاقة ارتباط سالبة بين نشاط الإنزيم عند مستوى ثانى أكسيد الكربون الجوى والمستوى المرتفع عن الجوى حيث ظهر أن نشاط البيرأكسيديز يتناقص فى التركيز العالى من ثانى أكسيد الكربون ويتزايد فى التركيز الجوى العادى. ويمكن تعليل زيادة نشاط البيرأكسيديز فى المستوى المنخفض بحدوث حث للنشاط الإنزيمى عند التركيزات المنخفضة حيث لم يتأثر البروتين ، بينما عند التركيزات المرتفعة يبدأ البروتين فى التأثر لذلك ينخفض نشاط البيرأكسيديز بوضوح كما يتضح عند تركيزات ٢٥٠، ٣٧٥ مليمولر NaCl بينما عند ٥٠٠ مليمولر NaCl يحدث ارتفاع أقل من المشاهد عند ١٢٥ مليمولر قد يعلل ذلك بوجود تأقلم

للنبات عند المستوى العالى من كلوريد الصوديوم خيث أن تعرضه للمستوى العالى من الملوحة قد يسبب مقاومة أولية ضد التأثير الملحى المتزايد ، كما أن انعكاس النشاط الإنزيمى تحت تأثير المستوى المرتفع من ثانى أكسيد الكربون يمكن تفسير ذلك بأن النبات تحت ظروف الإجهاد التأكسدى الملحى يقوم بغلق الثغور جزئيا وبالتالى نقص ثانى أكسيد الكربون الممتص ولكن نتيجة وجود مستوى مرتفع من ثانى أكسيد الكربون يستطيع النبات تحت تلك الظروف أن تزيد كمية ثانى أكسيد الكربون فى وحدة المساحة مما يمكنه من القيام بعمليات البناء والهدم تحت تلك الظروف بالرغم من الغلق الجزئى للثغور حيث يبدأ النشاط فى الارتفاع تحت تأثير الملوحة المتزايدة .

وتخلص النتائج أنه يمكن لنبات أستر أن يتحمل ظروف الإجهاد الملحى فى الجو العادى حتى ٢٥٠ مليمولر ، بينما فى المستوى العالى من ثانى أكسيد الكربون يمكنه التحمل حتى ٣٧٥ مليمولر حيث يبدأ النشاط الإنزيمى فى التأثر عند هذا المستوى للإجهاد التأكسدى الملحى . ويلاحظ أن تأثر البيرأكسيديز عند تركيز ١٢٥ مليمولر فى الجو العادى وعدم ظهور تأثراً اجهادياً فى التركيز المضاعف من CO_2 الجوى حتى مستوى ٢٥٠ مليمولر وبداية التأثر عند ٣٧٥ مليمولر يعنى أن النبات لايعانى إجهاداً تأكسديا ملحياً بدليل عدم تأثر البيرأكسيديز . ولذلك تم اختيار تركيز ٣٧٥ مليمولر حيث أنه يمثل نقطة التحول أو الانقلاب ، وأجرى تحليل بروتينى فى اتجاهين لعينة كنترول حديثة وكنترول بعد شهر من تعرض النبات للإجهاد التأكسدى وعينة معاملة ٣٥٠ مليمولر كلوريد الصوديوم عند المستوى الجوى العادى من CO_2 . أظهرت النتائج أن العينة الحديثة تحتوى على العديد من السلاسل الببتيدية والتى تظهر انتشارا فى درجات مختلفة من الـ pH بينما لم تعط العينة الكنترول الأخرى نفس العدد من السلاسل الببتيدية بل لوحظ حدوث تراكم وتجمع فى مدى ضيق من درجات الـ pH عند درجات حامضية وهى نفس النتيجة التى ظهرت فى العينة المعرضة للإجهاد الملحى التأكسدى مع حدوث اختفاء العديد من السلاسل الببتيدية فى العينة المعاملة .

وتشير الدراسة إلى الدور الذى يمكن أن تلعبه النباتات الملحية فى النمو تحت ظروف الملوحة المرتفعة وزراعتها فى الأراضى الملحية والاستفادة من تلك النباتات كعلف أو كمستخلصات لها أهمية علاجية ، كما أظهرت الدراسة الدور البيئى الذى يمكن أن تقوم به تلك النباتات فى امتصاص ثانى أكسيد الكربون الجوى تحت تلك الظروف الإجهادية وعدم تأثر النشاط الإنزيمى لها (البيرأكسيديز فى تلك الدراسة) حيث أنه من الإنزيمات الذى تتأثر بوضوح فى الإجهاد التأكسدى ، مما يعنى الدور الذى يمكن أن تساهم به فى نقص ظاهرة الإنبعاث الحرارى أو مايطلق عليه الـ green house effect .