Pertanika J. Trop. Agric. Sci. **29 (1 & 2)**: 57-66 (2006)

ISSN: 1511-3701

Paraquat (Methyl viologen) Toxicity in *Centella asiatica* Callus Cultures

NOR'AINI MOHD FADZILLAH, NORHAYATI YUSUF, [1]MARZIAH MAHMOOD, MISRI KUSNAN & SITI KHALIJAH DAUD

Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

E-mail: aini@fsas.upm.edu.my

Keywords: ***Centella asiatica*, paraquat, callus growth, cell viability, malondialdehyde, hydrogen peroxide**

ABSTRAK

Kajian telah dijalankan untuk mengkaji kesan rawatan paraquat (PQ) ke atas pertumbuhan, viabiliti, kandungan hidrogen peroksida dan malondialdehida di dalam kultur kalus Centella asiatica (CA03 dan CA09). Kalus dirawat dengan 50 µM PQ selama 5 hari di dalam medium cecair Murashige dan Skoog (MS). Pertumbuhan, viabiliti kalus dan juga kandungan hidrogen peroksida (H_2O_2) dan malondialdehida (MDA) ditentukan pada hari 0, 1, 2, 3 dan 5 rawatan. Berat basah dan berat kering kalus CA03 yang diberi rawatan adalah lebih rendah berbanding kalus kawalan. Bagi kalus CA09 pula, pertumbuhan kalus rawatan adalah lebih rendah berbanding kalus kawalan pada peringkat akhir tempoh rawatan. Walaupun terdapat perencatan pertumbuhan bagi kedua-dua CA03 dan CA09 yang diberi rawatan, perencatan berat basah sebanyak 36% bagi CA09 berbanding dengan kawalannya pada akhir tempoh rawatan adalah lebih tinggi dari CA03 di mana terdapat perencatan pertumbuhan berat basah sebanyak 18.2%. Penurunan peratus viabiliti sel juga adalah sangat ketara terutama pada kalus CA09 selepas dirawat dengan PQ. Walaupun kandungan MDA adalah lebih tinggi di dalam kalus CA03 berbanding CA09 yang diberi rawatan pada peringkat awal, ia menunjukkan corak perubahan yang menurun mengikut masa manakala kandungan MDA pada CA09 pula menunjukkan corak perubahan yang meningkat. Pada akhir tempoh rawatan, MDA pada CA09 adalah lebih tinggi berbanding CA03. Tambahan lagi, kandungan H_2O_2 pada amnya adalah lebih tinggi pada CA09 yang diberi rawatan berbanding CA03 kecuali pada hari ke 3. Kajian ini menunjukkan bahawa rawatan dengan PQ boleh meransang penghasilan MDA dan H_2O_2 dan juga merencatkan pertumbuhan dan juga viabiliti kalus. Kajian juga menunjukkan kalus CA03 adalah lebih toleran kepada PQ berbanding CA09.

ABSTRACT

The effect of paraquat (PQ) treatment on growth, cell viability, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels were investigated in the callus of two Centella asiatica accessions (CA03 and CA09). Callus of C. asiatica were treated with 50µM PQ for five days in Murashige and Skoog (MS) liquid medium. Callus growth, viability of the callus as well as H_2O_2 content and MDA levels were evaluated at 0, 1, 2, 3 and 5 days of treatment periods. Fresh weight and dry weight of treated calli were significantly lower in CA03 as compared to the untreated calli. In CA09, the growth of treated calli was significantly lower compared to controls at the later stages of the treatment period. Although decreases in growth were observed for both treated CA03 and CA09, the final reduction in fresh weight at 36% for CA09 compared to its control was much higher compared to CA03 with an 18.2% final reduction in fresh weight. PQ treatment also resulted in a marked decrease in the viability of the callus especially in CA09. Although MDA levels were significantly higher in treated CA03 as compared to treated CA09 at the early treatment stages, they showed a decreasing trend, while MDA levels in CA09 showed an increasing trend, which was significantly higher than that of CA03 at the end of the treatment period. In addition, H_2O_2 concentrations were generally higher in treated CA09 compared to treated CA03 except at day 3. This study indicated that PQ treatment can induce increases in levels of MDA and H_2O_2, associated with the decrease in growth and viability of the callus. Results also suggested that CA03 was more tolerant to PQ treatment as compared to CA09.

INTRODUCTION

Oxidative stress is very frequent in nature and is due to an increase of the reactive oxygen species (ROS). It is induced by several biotic and abiotic factors including phytotoxic chemical agents including non selective herbicides (e.g paraquat, PQ). The bipyridyl herbicides such as paraquat, also known as methyl viologen, with its active compound 1,1'-dimethyl-4,4'-bipyridylium dichloride and diquat are non-selective, quick acting herbicides, effective against grasses as well as most broad-leaved weed species (Calderbank and Slade, 1976). The major target of the bipyridyl compounds in PQ seems to be the chloroplast; PQ can accept one electron from photosystem I and the formed PQ radicals is rapidly oxidized under the catalyzation of metal ions, leading to the formation of superoxide radicals. In further reactions, various ROS e.g H_2O_2 and hydroxyl radicals are generated (Lorenzini *et. al.*, 2002). The hydroxyl radical generated will rapidly react with membrane unsaturated fatty acid leading to membrane damage, reduction in CO_2 uptake and degradation of chloroplast and pigments (Kirtikara and Talbot, 1996). These ROS are efficiently scavenged by a series of enzymes and quenching systems such as superoxide dismutase, enzymes in ascorbate glutathione cycle, ascorbate, glutathione and membrane bound α-tocopherol (Suntres, 2002).

Oxidative stress is also involved in loss of viability of plants exposed to a variety of environmental stress. The 2,3,5-triphenyl tetrazolium chloride (TTC) assay was used as a viability assay for callus exposed to various concentrations of PQ. The production of MDA and changes in cell conductivity have frequently been used as sensitive markers for herbicides' action in plants (Peleg'*et al.*, 1992).

Centella asiatica is commonly used as a vegetable or eaten raw as an 'ulam' (Malay salad). Apart from being a nutritious plant,'*C. asiatica* is also believed to have many healing properties, conferring a wide range of beneficial effects and is treated as a valuable medicinal plant in Chinese traditional medicine and classical Indian Ayurvedic medicine. Research has demonstrated that *C. asiatica* is a rich source of natural antioxidants. These antioxidants are scavengers of ROS and inhibitors of lipid peroxidation and thus, can protect and defend cells against damage by the ROS (Subramaniam *et al.*, 1998).

The main objectives of this study were to determine the effect of PQ treatments on growth, cell viability, H_2O_2 and MDA levels of two *C. asiatica* callus cultures i.e CA03 and CA09.

MATERIALS AND METHODS

Callus Initiation and Maintainance

Sterile leaf explants of *C. asiatica* were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) and kinetin. The cultures were maintained by regular subculturing at 10 day intervals onto fresh medium. All cultures were incubated in 12h/12h (light/dark) photoperiod under cool, white fluorescent lamps at 27 ± 2^{o}C.

PQ (1,1'-dimethyl-4,4'-bipyridylium dichloride) Treatment

Callus pieces were transferred to MS medium containing 50 μM PQ. PQ is heat-stable and for all experiments, was added to the medium prior to autoclaving. Callus growth, cell viability, H_2O_2 content and MDA levels were assayed at 0, 1, 2, 3 and 5 days of treatment periods.

Callus Growth and Viability

Treated callus were washed with distilled water and weighed immediately for fresh weight. For dry weight, callus were dried in an oven at 50^{o}C for 2 days. The 2,3,5-triphenyltetrazolium chloride (TTC) assay was used to estimate the proportion of viable cells after PQ treatments. The absorbance of the supernatant was determined at 485 nm (Towill and Mazur, 1974).

MDA Assay

MDA concentration was determined by the thiobarbituric acid (TBA) reaction, based on the method by Heath and Packer (1968) with slight modification by Hodges *et al.* (1999).

H_2O_2 Determination

H_2O_2 assay was done following the method of Velikova *et al.* (2000).

RESULTS AND DISCUSSION

The changes in growth of *C. asiatica* callus cultures treated with 50 μM PQ are shown in *Figs. 1* and *2*. Decreases in fresh weight and dry weight were observed 24 hours after treatment with PQ in both accessions. The reduction in growth was greater in CA09 as compared to CA03 especially after 2 days of treatment (*Figs. 1C* and *2C*). In CA03, the growth of treated callus were significantly lower ($p<0.05$) than the control (*Figs. 1A* and *2A*), but in CA09 the growth of treated callus were only significantly lower than its control ($p<0.05$) at the later stages of treatment periods (*Figs. 1B* and *2B*). This however was due to the sharp decrease in fresh weight of the control CA09 callus at day 2 which increased slightly thereafter. Therefore, although significant differences in fresh weight for CA09 only occurred at the later stages of treatment period, this was due to the low fresh weight values of its control. A comparison of the actual final reduction in growth between CA03 and CA09 showed a 36% reduction in CA09 while CA03 showed 18.2% reduction of growth compared to their respective controls (*Figs. 1A* and *1B*). The similar dry weight values of treated CA03 and CA09 showed that there were no differences in terms of actual organic matter content in the two accessions (*Fig. 2C*). However, the lower reduction in growth of CA09 is probably due to the significantly lower percentage of viability compared to CA03 (*Fig. 3C*). *Fig. 3* demonstrates that PQ treatment resulted in a marked decrease in the viability of the callus especially in CA09. After 24 hours treatment with PQ, only 5% of CA09 callus were still viable while only 1.5% of the cells were viable after 5 days of treatment (*Fig. 3C*). The viable proportion of the callus were significantly higher ($p<0.05$) in controls as compared to the treated callus in both accessions (*Figs. 3A* and *3B*). The results of Wong (2000) also suggest that PQ at a low concentration (0.02 mg/l) can significantly inhibit the growth, photosynthetic rates and chlorophyll content of *Scenedesmus quadricauda* Berb 614. Clearly, PQ is more effective in decreasing the growth and cell viability of CA09 callus than CA03. Results thus indicate that PQ could induce oxidative stress in *C. asiatica* callus cultures.

The condition of oxidative stress induced by PQ resulting in peroxidation of membrane lipids in the *C. asiatica* cultures is clearly indicated by the increased MDA levels in both accessions compared to their respective controls except for days 1 and 3 in CA09 callus (*Figs. 4A* and *4B*). MDA levels were initially significantly higher ($p<0.05$) in CA03 as compared to CA09 especially up to 2 days of treatment periods. However, longer treatment period decreased the MDA levels in CA03 and increased the MDA levels in CA09 (*Fig. 4C*). At the end of the treatment period, MDA levels in CA09 were significantly higher ($p<0.05$) than that of CA03.

H_2O_2 concentrations between control and treated cultures of CA03 and CA09 were not significantly different except for day 3 for CA03 where the H_2O_2 concentrations in the treated callus were significantly higher ($p<0.05$) than its control (*Fig. 5A*). The treated CA09 callus did not show any increases in H_2O_2 concentrations after day 2 (*Fig. 5B*). This could be due to a compromised antioxidative defense in CA09 which although may have increased levels of superoxide radicals ($O_2^{\cdot-}$) production due to the PQ-induced oxidative stress, was not able to dismutate these radicals to H_2O_2. CA03 on the other hand was probably still capable of modulating its antioxidative defense resulting in the sudden burst of H_2O_2 production which was subsequently neutralized to less harmful forms. This was reflected in the decreased levels of H_2O_2 after day 3 (*Fig.*

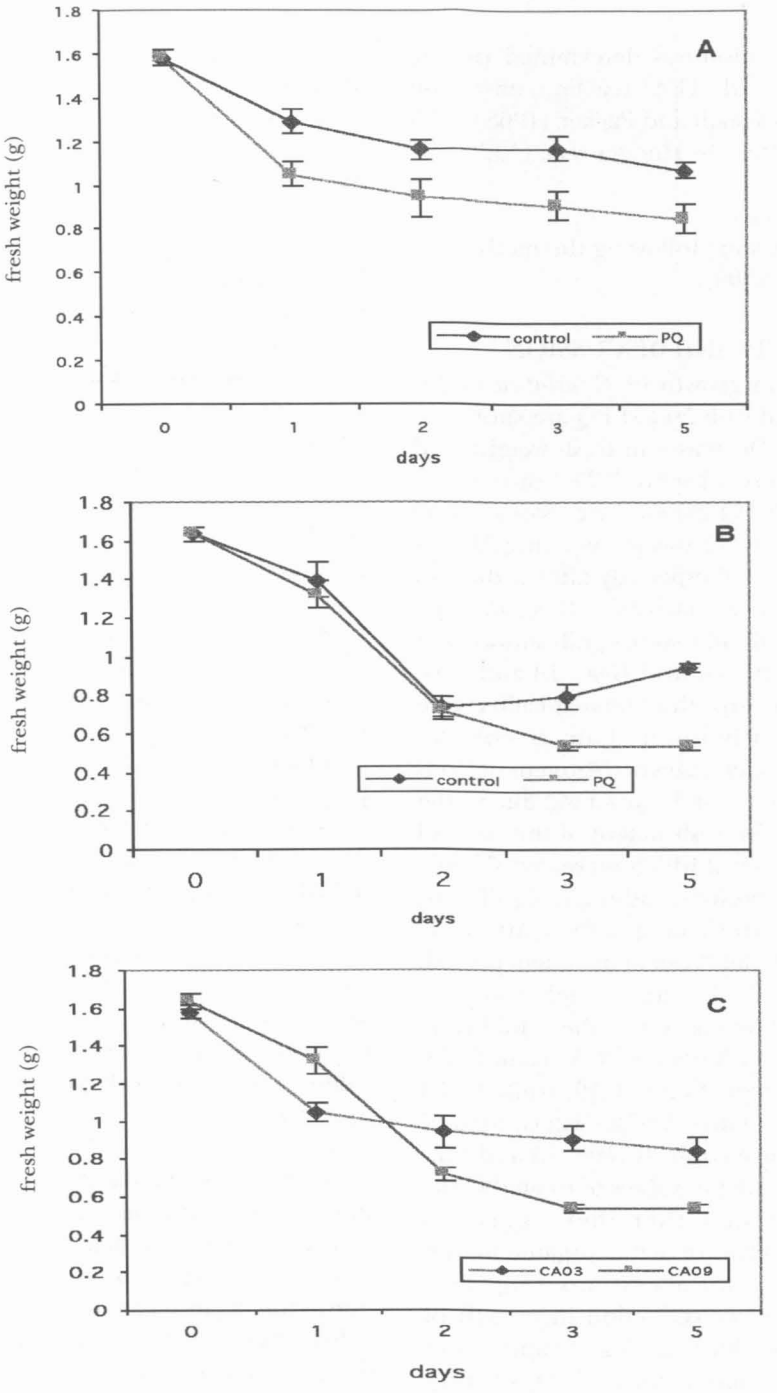
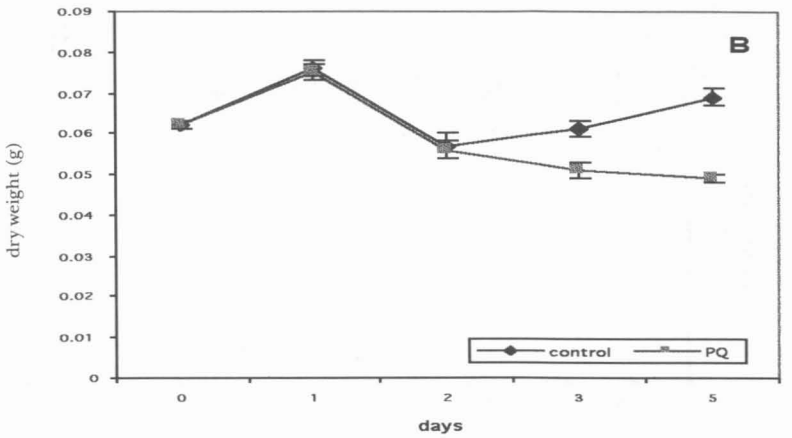
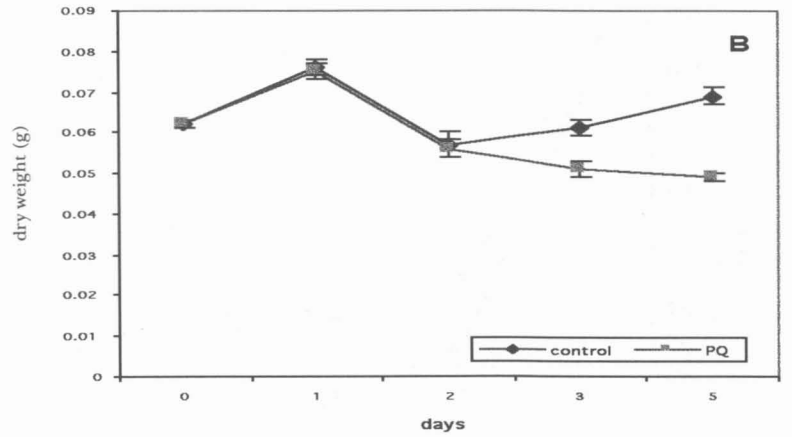


Fig. 1: Fresh weight (g) of C. asiatica *callus culture:*
A) CA03 callus
B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)





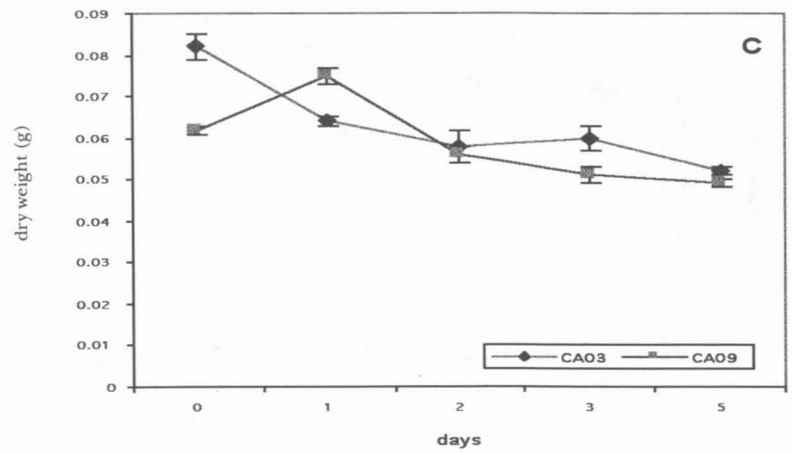


Fig. 2: Dry weight (g) of C. asiatica *callus culture:*
A) CA03 callus
B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

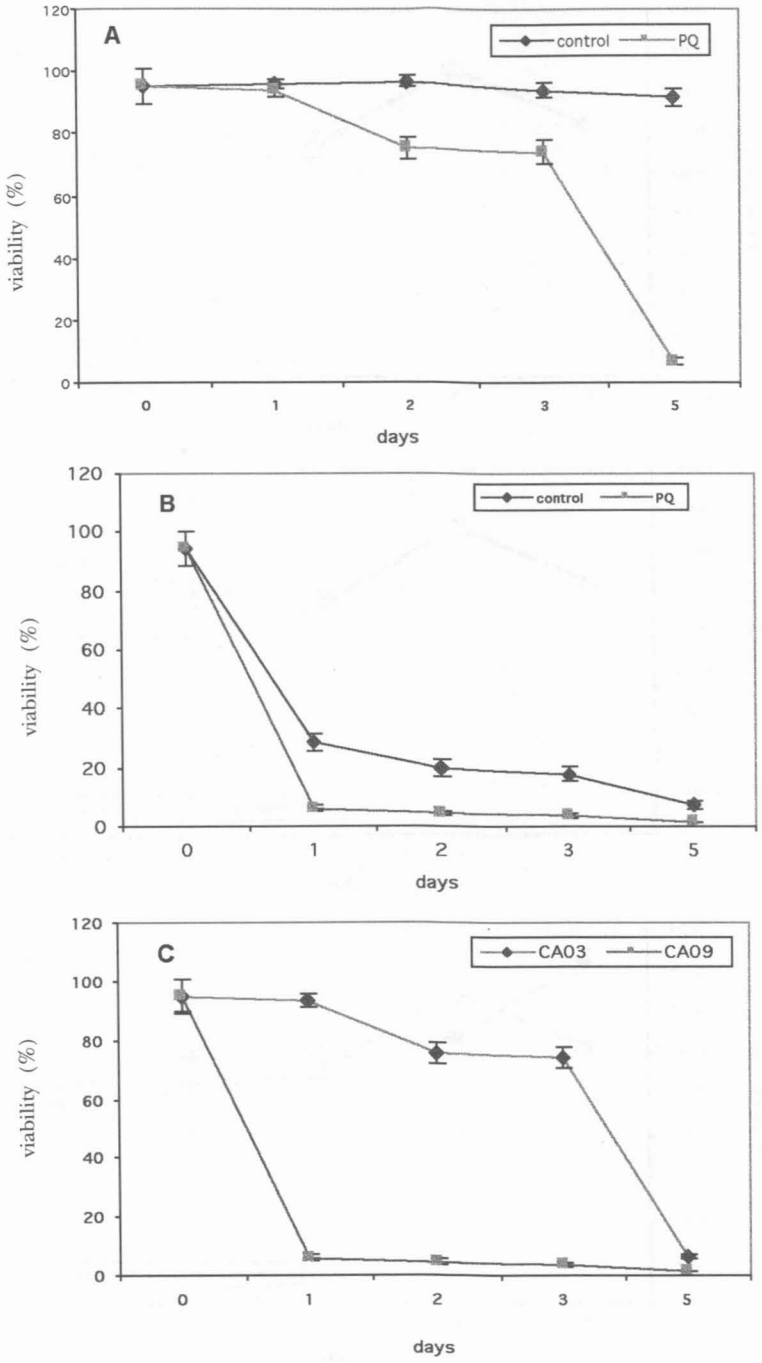


Fig. 3: Viability (%) of C. asiatica *callus culture:*
A) CA03 callus
B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

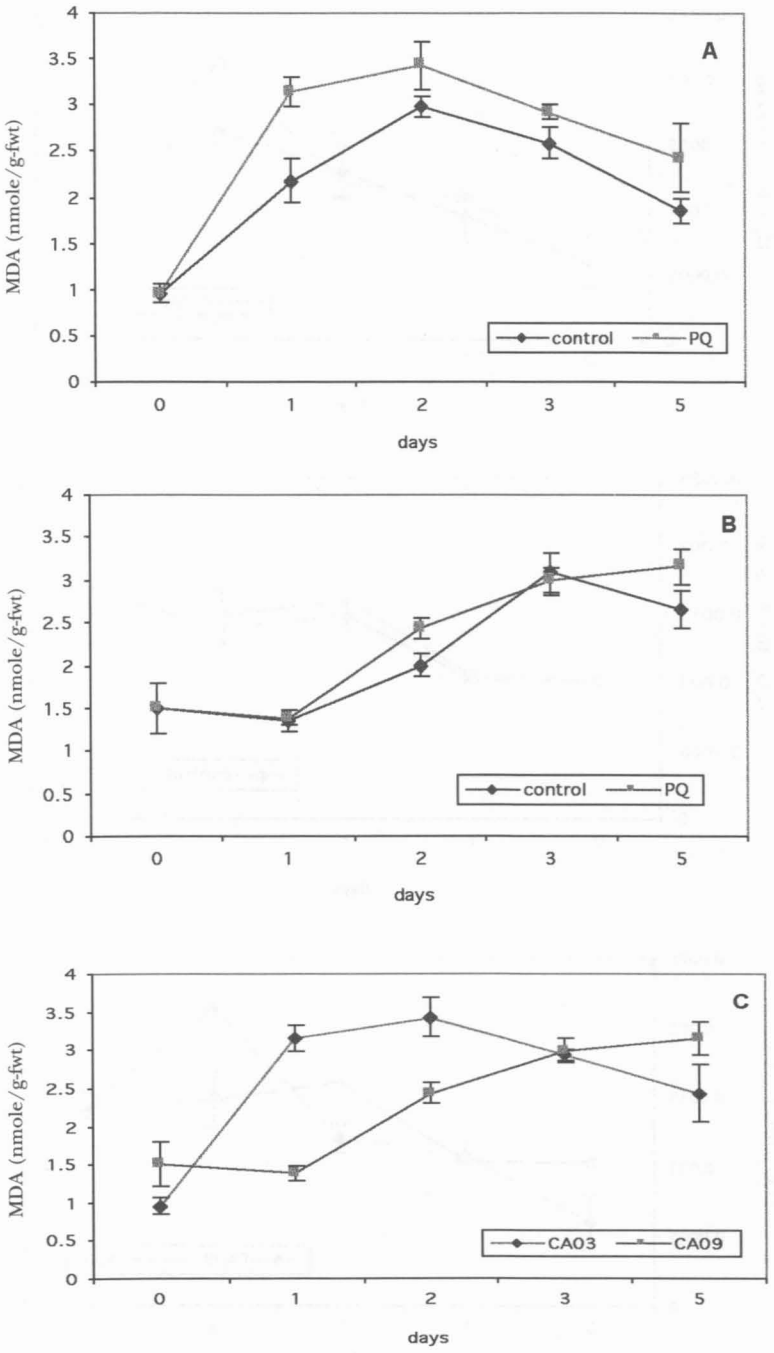


Fig. 4: Malondialdehyde (MDA) concentrations of C. asiatica *callus culture:*
A) CA03 callus B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

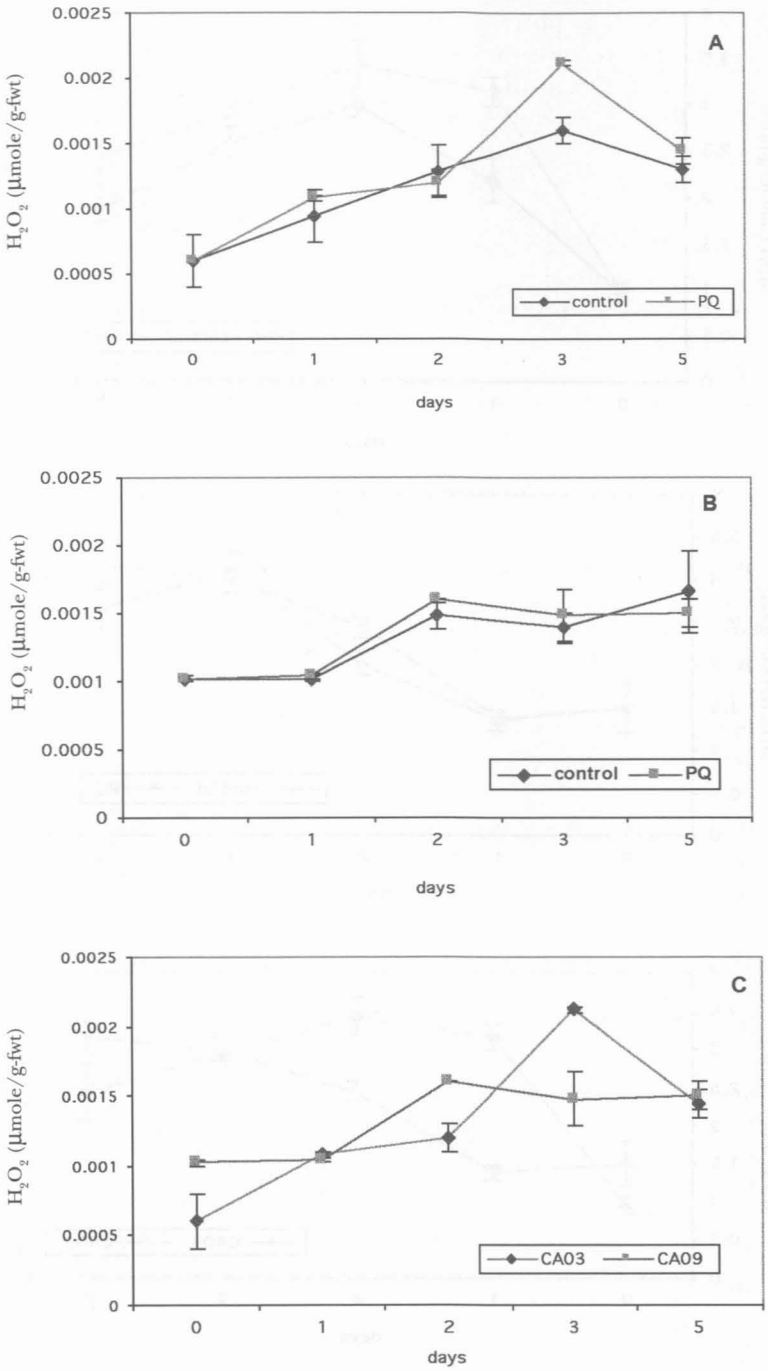


Fig. 5: Hydrogen peroxide (H_2O_2) concentrations of C. asiatica *callus culture:*
A) CA03 callus B) CA09 callus
C) CA03 and CA09 calli (PQ treatment)
Vertical bars represent standard errors (n=5)

5C). Accumulation of H_2O_2 is potentially harmful since it can lead to oxidative damage and loss of structure and function. The decrease in H_2O_2 and MDA concentrations in CA03 callus especially after 3 days of treatment period may be due to the higher antioxidant activity which had enhanced oxidative stress tolerance. H_2O_2 is an active oxygen species which can also react with superoxide radicals to form more powerful oxygen free radicals and hydroxyl radical in the presence of trace amounts of Fe or Cu (Bowler *et al.*, 1992). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids (Halliwell, 1987). These results are in agreement with the hypothesis that ROS and lipid peroxidation are major contributors to PQ toxicity (Hart and DiTomaso, 1994). Hutchison (1979) also demonstrated that PQ stimulated both H_2O_2 and MDA production in leaf and thylakoids of spinach.

CONCLUSIONS

The results obtained showed that PQ treatment can induce oxidative stress in *C. asiatica* callus cultures. There was a direct relationship between lipid peroxidation and ROS production since MDA levels increased in response to PQ treatment. In addition, PQ inhibited the growth and viability of the callus. Results also suggest that CA03 was more tolerant to PQ treatment as compared to CA09.

REFERENCES

Bowler, C., Van Montagu, M. and Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology, 43,* 83-116.

Calderbank, A. and Slade, P. (1976). Diquat and paraquat. In P.C. Kearney and D.D. Kaurman (Eds.), *Herbicides-chemistry, degradation and mode of action* (p. 501-540). New York: Marcel-Dekker.

Halliwell, B. (1987). Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chemistry Physics and Lipids, 44,* 327-340.

Hart, J.J. and DiTomaso, J.M. (1994). Sequestration and oxygen radical detoxification as mechanisms of paraquat resistance. *Weed Science, 42,* 277-284.

Heath, R.L. and Packer, L. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives Biochemistry Biophysics, 125,* 180-198.

Hodges, D.M., DeLong, J.M., Forney, C.F. and Prange, R.K. (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta, 207,* 604-611.

Hutchison, J.M. (1979). Hydrogen peroxide production and lipid peroxidation induced by paraquat in isolated cells and chloroplasts of spinach (*Spinacea oleracea* L.). (Ph.D. Dissertation, 100 p., University of California, 1979).

Kirtikara, K. and Talbot, D. (1996). Alteration in protein accumulation, gene expression and ascorbate-glutathione pathway in tomato (*Lycopersicon esculentum*) under paraquat and ozone stress. *Journal of Plant Physiology, 148,* 752-760.

Lorenzini, G., Stringari, S. and Nali, S. (2002). The absence of cross tolerance between ozone and paraquat : the case of *Conyza bonariensis. Phyton., 42,* 89-96.

Miller, O.K. and Hughes, K.W. (1980). Selection of paraquat-resistant variants of tobacco from cell cultures. *In Vitro., 16*(12), 1085-1091.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum, 15,* 473-497.

PELEG, I., ZER, H. and CHEVION, M. (1992). Paraquat toxicity in *Pisum sativum*: Effects on soluble and membrane-bound proteins. *Physiologia Plantarum, 86,* 131-135.

SUBRAMANIAM, V., ADENAN, M.I. and AHMAD, A.R. (1998, December). Antioxidant 'ulam' to fight free radical. *FRIM in Focus,* 3-5.

SUNTRES, Z.E. (2002). Role of antioxidant in paraquat toxicity. *Toxicology, 180*(1), 65-77.

TOWILL, L.E. and MAZUR, P. (1974). Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. *Canadian Journal of Botany, 53,* 1097-1102.

VELIKOVA, V., YARDANOV, I. and EDREVA, A. (2000). Oxidative stress and some antioxidant systems in acid rain treated bean plants: Protective role of exogenous polyamines. *Plant Science, 151,* 59-66.

WONG, P.K. (2000). Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauda* Berb 614. *Chemosphere, 41,* 177-182.