Phytochemistry, Vol 28, No 4, pp 1003 1006, 1989
Printed in Great Britain

0031 9422/89 $3 00+0 00

MEMBRANE DAMAGE CAUSED BY EXPOSURE TO *t*-BUTYL HYDROPEROXIDE

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(*Received* 15 *August* 1988)

Key Word Index—*Brassica rapa*, turnip, *Ipomoea batatas*, sweet potato, membrane damage, respiration, lipid hydroperoxide

Abstract—As well as red beet discs, turnip and sweet potato storage tissues exposed to *t*-butyl hydroperoxide (*t*-BHP) showed leakage of several cellular components including potassium ions, reducing sugars and UV-absorbing materials. Even at the beginning of the eight hr-treatment period, leakage of potassium ions was accompanied by a decrease in respiratory activity. At the time when the respiration of turnip discs started to decrease, accumulation of lipid hydroperoxides was detected by four different methods Just after *t*-BHP treatment started incorporation of *t*-BHP was observed and the amount increased linearly to a maximum after *ca* four hr and then decreased gradually. This disc experiment suggests that *t*-BHP, a model compound of oxygen stress, is incorporated into plant tissues and may cause directly or indirectly lipid peroxidation in various biomembranes which leads to a leakage of cellular components and a decrease in respiratory activity

INTRODUCTION

The most common and visibly apparent characteristic of plant tissue senescence is the wilting of leaves and the shrinkage of fruits. This is a result of an increase in water loss from the tissue. Age-related water loss is found to be accompanied by an increase in the rate of leakage of other small molecules, such as ions, sugars, water-soluble pigments and amino acids, which could cause some abnormal metabolism in those cells and thus lead to injury and death [1, 2]. Although such studies strongly suggest the breakdown of membrane structure during senescence, the physiological and/or biochemical nature of the processes underlying the membrane changes are not well understood at present Investigating the mechanism of membrane leakage is important for understanding plant senescence at the molecular level

We have studied red beet discs exposed to *t*-butyl hydroperoxide (*t*-BHP) as a model system for examining the precise changes in cellular membranes at the onset of leakage [3, 4] In this experimental system the time when betacyanin leakage occurred was easily controlled by changing the concentration of *t*-butyl hydroperoxide (*t*-BHP) and the duration of the treatment. As well as betacyanin, potassium ions, reducing sugars, UV-absorbing materials and proteins leaked out from red beet discs after *t*-BHP treatment, but the timing of these leakages is likely to be different depending on molecular size [3].

The present study was designed to assess further the response of other plant tissues (sweet potato and turnip) to *t*-BHP exposure and confirm that it is not a phenomenon confined only to red beet discs. It was also aimed at examining the mechanism of leakage and reduced respiration during *t*-BHP treatment.

RESULTS

Leakage of cellular components from turnip and sweet potato discs exposed to t-*BHP*

Throughout 10 hr after *t*-BHP treatment (10 mM, 8 hr) the efflux of potassium ions, reducing sugars and UV-absorbing materials was followed in the incubation medium in which four turnip or sweet potato discs were immersed (Fig. 1). As found from discs of red beet storage tissues [3], little leakage was observed from the control discs (without *t*-BHP treatment) of the both plant tissues, but from the *t*-BHP treated ones a considerable leakage of cellular components was found 4–6 hr after the transfer of treated discs to a new medium without *t*-BHP. The concentrations in the medium increased gradually with time and after 10 hr from sweet potato discs *ca* 90% of both the total potassium ions and total UV-absorbing materials leaked out into the incubation medium; from turnip discs 85% of the total reducing sugars, 55% of the UV-absorbing materials and 65% of the potassium ions leaked out

Other tissues such as egg plant fruit, radish root, potato tuber, white gourd fruit and chayote fruit responded to *t*-BHP in a similar fashion showing leakage of reducing sugars and potassium ions (data not shown).

Potassium ion leakage and decrease in respiratory activity in discs exposed to t-*BHP*

In our previous paper [3], leakage of potassium ions was observed *ca* three hr after exposure of the discs to *t*-BHP, preceding the leakage of other cellular components. The leakage of potassium ions and the decrease in

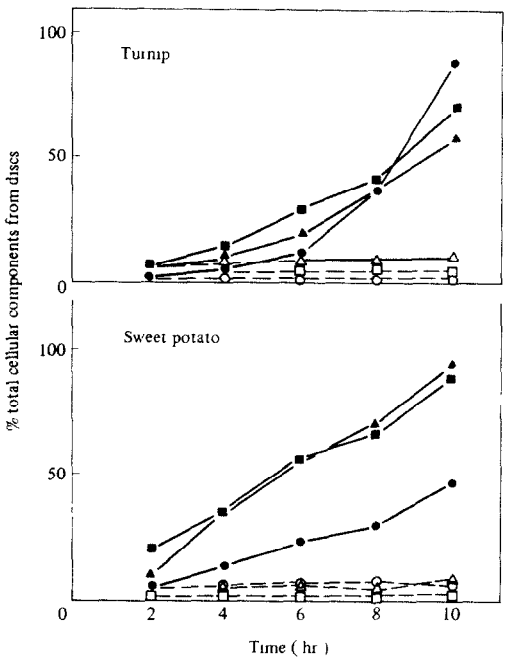

Fig 1 Leakage of cellular components, reducing sugars (-●-), UV-absorbing materials (-▲-) and potassium ions (-■-), from root discs of turnip and sweet potato incubated with *t*-BHP (10 mM) Leakage was examined in a *t*-BHP free medium after treatment (10 mM and 8 hr) Open symbols represented loss of components from discs without treatment

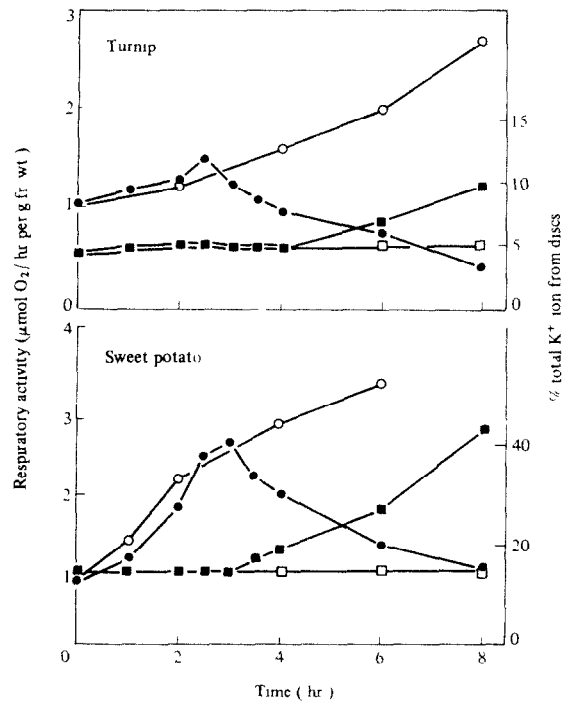

Fig 2 Leakage of potassium ion (-■-) from root discs of turnip and sweet potato and changes in the respiration (-●-) during *t*-BHP treatment (10 mM) Open symbols represent leakage from control discs incubated without *t*-BHP

respiratory activity of turnip and sweet potato discs during eight hr of *t*-BHP treatment is shown in Fig 2 The potassium leakage was different between the control media of the two tissues (*ca* 5 and 18%, respectively) and no change was recognized throughout the incubation period (10 hr). From *t*-BHP treated discs a release of potassium ions was observed 3-4 hr (sweet potato) and 4-6 hr (turnip) By the end of the eight hr treatment period more than 40% of the total potassium ions had leaked out from the discs of sweet potato Leakage from turnip discs was considerably smaller (*ca* 10%)

The respiratory activities of control discs of turnip and sweet potato increased gradually and reached 3 5 μmol O_2/hr/g fr wt and 1 8 μmol O_2/hr/g fr wt after six hr incubation Discs treated with *t*-BHP showed a sudden decrease in activity after 3-3 5 hr (sweet potato) and 2 5-3 hr (turnip) and then continued to decrease At the end of eight hr-treatment the treated discs of turnip showed only 11 5% of the respiratory activity of control discs and for sweet potato this decreased to 30% of the control after six hr

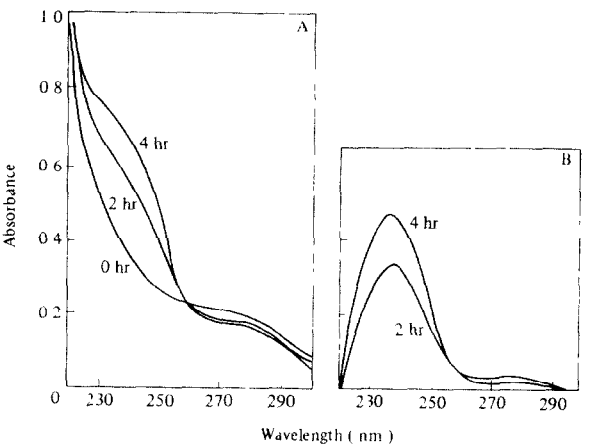

Fig 3 UV-absorption spectra (A) and differential spectra (B) of lipids extracted from discs of turnip roots 0, 2 and 4 hr after *t*-BHP treatment

Production of lipid hydroperoxides in mitochondrial membranes from turnip discs exposed to t-*BHP*

Crude lipids extracted from a mitochondrial fraction which was prepared from turnip discs by a centrifugation method were investigated by four procedures to detect lipid hydroperoxides The UV spectrum of the lipids extracted from *t*-BHP treated discs of turnip after 2 and 4 hr is shown in Fig 3 Maximum absorption was observed at *ca* 235-240 nm in the difference spectra from both samples which suggested that it was attributable to conjugated dienes of lipid hydroperoxides The differential absorbance increased with time

Three other procedures were used to determine lipid hydroperoxides in the crude lipid extracts from mitochondrial fractions of control and treated discs of both tissues Apart from the TBA method (0 8 nmol/g/fr wt) two other procedures, iodide-oxidation and Hb-MB, showed only trace amounts were detected in crude lipids of the 0 hr sample A gradual accumulation of lipid

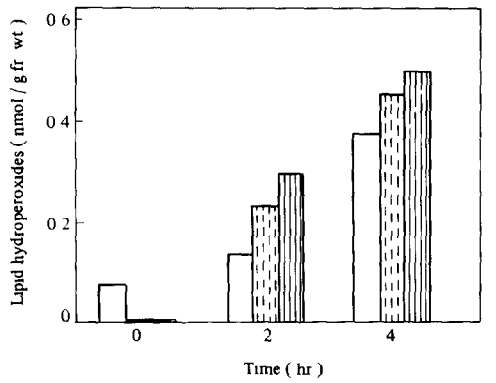

Fig 4 Concentrations of lipid hydroperoxides in mitochondrial lipids from turnip root discs incubated with *t*-BHP (10 mM) Determined by the TBA (□), iodide-oxidation (▤), and haemoglobin-Methylene Blue (▥) methods

hydroperoxides was then observed (Fig 4). In lipid samples prepared from discs four hr after *t*-BHP treatment, the levels were 0 37, 0 44 and 0 50 nmol/g/fr. wt, respectively, from the TBA, iodide-oxidation and Hb-MB methods This pattern of hydroperoxide increase resembled that of the differential absorbance at 235–240 nm

Incorporation of t-*BHP into turnip discs during the* t-*BHP treatment*

Discs were homogenized in methanol and the amount of the *t*-BHP incorporated was determined by two different analytical procedures, iodide-oxidation and GC. The amount of *t*-BHP taken up as measured by iodide-oxidation increased linearly, reaching a maximum (2 3 μmol/g/fr wt) after 4 hr and then gradually decreased to 1 2 μmol/g/fr wt after 10 hr (Fig. 5). A similar pattern of incorporation was found using the GC technique (maximum value. 1.92 μmol/g/fr wt)

DISCUSSION

It is evident that the phenomenon of membrane leakage induced by *t*-BHP is not a special one observed only in red beet discs Other cellular components leak out as well as betacyanin. Since several fruit tissues also responded to the *t*-BHP treatment similarly, *t*-BHP-induced leakage is considered to be a general phenomenon among plant tissues other than green leaves. The compound also causes a remarkable decrease in respiratory activity These observations suggest that disorder of some cellular membranes occurs in the cells and thus leads to a decrease in respiration and an increase in membrane permeability during *t*-BHP treatment If this is correct the system using *t*-BHP and plant tissue discs may be useful for examining membrane disorders or damage which are presumed to occur at the onset of plant senescence or other physiological injury

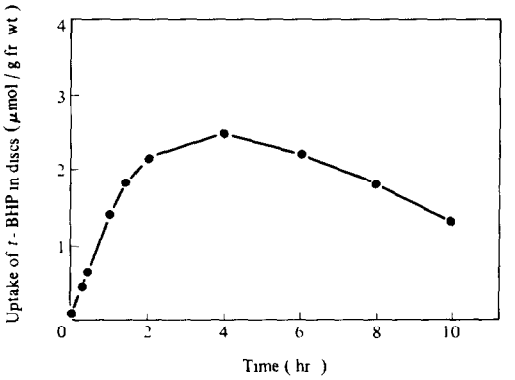

Fig 5 Uptake of *t*-BHP into root discs of turnip during the treatment

It is interesting to note that low levels of lipid hydroperoxides (2 7–3 3 nmol/mg mitochondrial lipids) is likely to be responsible for the changes in respiratory activities in *t*-BHP treated tissues Formation and accumulation of lipid hydroperoxides are reported to change the physical properties and function of bio-membranes or artificial membranes [5–12]

t-BHP (3.9%, 1.93 μmol) was incorporated into the discs of turnip (fr. wt; 0.88 ± 0.04 g) from 5 ml of the incubation medium including 10 mM *t*-BHP (50 μmol) after two hr incubation at 20° Although at the present time there is no evidence to support whether the chemical acts on the cellular membranes directly or indirectly, Trotta *et al.* [13, 14] reported that in human red blood cells, *t*-butoxyl radicals derived from *t*-BHP in a reaction which was catalysed by chelated Fe^{3+} ion, haemoglobin, attacked red blood cell membranes resulting in haemolysis. It is suggested that a similar mechanism could be induced by chelated Fe^{3+} ion in plant tissues and such *t*-butoxyl radicals could easily cause lipid peroxidation in biomembranes.

Since our previous paper [4] reported that some radical scavengers effectively prevented betacyanin leakage from red beet discs, some parts of incorporated *t*-BHP are transformed to *t*-butoxyl radicals in the plant cells The results of this model experiment give suggestions that a small amount of radicals produced at the onset of plant senescence or a physiological disorder such as chilling injury may play an important role in producing abnormal secondary metabolisms and symptoms

EXPERIMENTAL

Tubers of sweet potato (*Ipomoea batatas* Lam) and turnip (*Brassica rapa* L) were obtained fresh from a local market Discs of *ca* 1.5 mm thickness and 13 mm diameter were prepared from the storage tissues, washed twice with 10 mM MES–NaOH buffer (pH 6) containing 0.2 M mannitol and transfered to 50 ml conical flasks containing 5 ml of the same buffer with 0.2 M mannitol and streptomycin (1 μg/ml) Four discs (fr wt turnip discs 0 84 ± 0 04 g, sweet potato discs 0 88 ± 0 04 g) in a flask were treated with 10 mM *t*-BHP at 20° in the dark for various times, usually 8 or 10 hrs, followed by washing with buffer containing mannitol and streptomycin but no *t*-BHP Discs were then incubated in the same medium or a test solution

Measurement of leakage of cellular components was carried out by determining the amounts released from discs after or during *t*-BHP treatment Reducing sugars were determined by the method of ref [15], potassium ions by an atomic absorption method and UV-absorbing materials by a spectrophotometric method (*A* at 260 nm)

Respiration of discs was measured as O_2 uptake polarographically at 20° with a Clark-type Pt electrode Lipid hydroperoxides in lipid extracts of crude mitochondrial fractions,

which were prepared by a centrifugation method [16], was determined by four methods, iodide-oxidation [17], A of conjugated dienes at 235 nm [18], haemoglobin–Methylene Blue [19, 20] Malonaldehyde derived from lipid hydroperoxides was assayed by the thiobarbituric acid method as described in ref [21] The amount of t-BHP incorporated into discs during t-BHP treatment was determined by two procedures, GC and iodide-oxidation [17] GC conditions fused silica column (0 24 mm id × 50 m) coated with PEG-20M, column temp 70° (5 min) to 110° at 5°/min R_ts of t-BHP and t-butyl alcohol, were 16 6 and 11 4 min, respectively All experimental values are the mean of three determinations in leakage, lipid hydroperoxides and t-BHP uptake expts, or of two determinations in respiration measurements Expts were repeated at least twice

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