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Petal Senescing Processes Monitored by Dynamic States of Water in Orchid Plants Exposed to Exogenous Ethylene

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Ethylene induces premature senescence of orchid flowers when plants are particularly transported with fruits and vegetables stored nearby. Exogeneous ethylene regulated a complex physiological changes of petals in intact flowering clones of *Dendrobium phalaenopsis* during seven days; decrease in distance between petals, water content, NMR relaxation times (T_1s and T_2s) and degree in vital staining, while increase in ion leakage and translucence. Petal wilting and decrease in water content in florets were closely correlated with loss in viability of petal parenchyma tissues adjacent to the vascular bundles in the ethylene-exposed orchid flowers. Highly mobile water measured by T_1 values of the long fraction in the petals was considered to free water derived from intact vacuoles. The loss of membrane integrity and free water of the orchid petals can be modulated by ethylene. After ethylene exposure treatment, the free water gradually decreased at 5 d. Additionally, T_2s of the short fraction in the petals markedly decreased at 5 d after the ethylene exposure and they reached to 10 ms at 7 d. From these results, the decrease in the T_1 and T_2 of the orchid petals due to ethylene exposure indicated decreased vacuolar water, followed by turgor loss and increased bound water, thus decrease in diffusion of substrates and metabolism.

Keywords : flower senescence, orchid (*Dendrobium phalaenopsis*), NMR relaxation times (T_1, T_2), water compartment

INTRODUCTION

Plants vary widely in their sensitivity to ethylene and it can hasten an onset of rise and a time to senescence both in fruits and flowers of some plant families (O'Neill, 1997; Hilioti et al., 2000 references therein). Orchid blossoms fade prematurely if they are gassed with ethylene, pollinated or treated with an auxin. The unpollinated intact orchid flowers are long-lasting and may live for up three month (Stead, 1992), while pollination is rapidly followed by the appearance of visible se-

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nescence symptoms, which are detectable within 24 h. Wilting of the flowers of *Dendrobium* sp. was accompanied by a loss of water from cells of the upper layer of the petals, leading to their upward folding 24 h (Porat et al., 1994). Applications of ethylene on orchids induce reactions similar to that of pollination and thereby it increased the ethylene-sensitivity (Ketsa and Rugkong, 2000). Ethylene caused petal abscission in *Pelargonium* cultivars (Cameron and Reid, 2001; Jones et al., 2001), and ethylene prevented water uptake to petals of *Dendrobium* sp (Ketsa and Rugkong, 1999).

Since many metabolic processes such as enzymatic reactions, transportation and accumulation of materials occur in the cytosol, water in living tissues is considered to play an important role in their physiological condition. Therefore, the physical states of water reflect cellular activity. Nuclear magnetic resonance (NMR) relaxation times, such as spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2), are used as indicators of dynamic states of water in biological tissues since they reflect the motion of water molecules (Farrar and Becker, 1971). Mobility and characteristic of cell-associated water is closely related to the condition of the cells, NMR images represent physiological maps of the plant tissues (Ishida et al., 1997, 2000; Kano et al., 1997). Furthermore, T_1 and T_2 of water protons have been applied to the studies of higher plant tissues exposed to thermal stresses (Kaku et al., 1985; Abass and Rajashekar, 1991; Iwaya-Inoue et al., 1993; Yoshida et al., 1997; Maheswari et al., 1999; Iwaya-Inoue et al., 2004). Senescence regulates a complex syndrome of developmental events in many flowers. The process of petal senescence was accompanied by decrease of water content and free water, turgor and protein content, and increased ion leakage in gladiolus and cut tulip flowers categorized as non-ethylene-sensitive plants (Iwaya-Inoue and Nonami, 2003 references therein).

Exogenous ethylene induces premature senescence of orchid flowers when plants are particularly transported with fruits and vegetables stored nearby. Although it has been shown that ethylene induce a set of consequential events in cut flowers of *Dendrobium* sp. (Nadeau et al., 1993; Porat et al., 1994), there is a few study on senescence of potted flowering plants exposed to ethylene (Serek and Sisler, 2001). In this study, we have characterized senescing processes of petals, especially dynamic states of tissue water in potted orchid plants exposed to ethylene.

MATERIALS AND METHODS

Plant materials

Figure 2 A-D shows an appearance of *Dendrobium phaelenopsis* cv. Linlin used in this study. Fifty pots of this flower were obtained from an orchid farmer in Maebaru City, closed to Fukuoka City, Fukuoka Prefecture, Japan. The individual flower stalk had approximately 10 open florets. Two lateral petals were used as following experiments.

Experimental conditions

The two growth cabinets indicating capacity 1.5 m^3 (Koito GC, Koito Industries, Ltd., Japan) represented two conditions, ethylene-exposure and control, respectively. 25 pots of flowering orchid plants were placed in the growth cabinets fitted with two gas sampling ports at upper and lower position, respectively. To uniformize the gas concentration inside, a ventilator was set to assure gas circulation inside. Each growth cabinet was kept at 27°C and relative humidity of 80 to 90% RH, under 14 h of illumination (100 μmol m^{-3} s^{-1}). Ethylene concentrations in the growth cabinets were measured by a gas chromatograph (GC 390; GL Science Co., Ltd., Japan), once in 90 min after ethylene injection, and once a day in other times. The samples were stored in each growth cabinets until the end of experiment.

For ethylene-exposure treatment, on both day 0 and day 1, 50 mL of ethylene was applied by introducing it through an injection port. On the other hand, the control plants were remained without ethylene. It was ascertained that the flowers in ethylene-exposure were exposed to over 10 ppm

of ethylene for two to three hours in these two days while the ethylene concentration of the control remained 0 ppm in the whole period of the storage.

Experimental procedures and wilting score

For the quality evaluations and measurements during their storage period, three pots for the

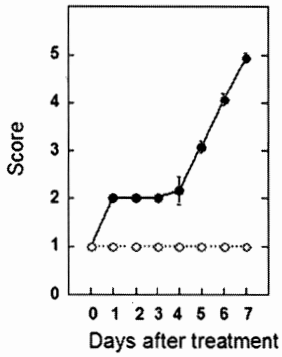

Fig. 1 Scores of petal senescence in orchid (*Dendrobium phalaenospis* cv. Linlin) exposed to ethylene during senescing processes. Life stages of a floret were defined by the following five degree of wilting: 1, fully opened; 2, turned upside down and drooped; 3, slightly wilted whole petal; 4, severely wilted whole petal; 5, severely wilted and browned whole petal. Control plants (○), Ethylene-exposed plants (●). Symbols represent mean values±standard error (n=5–7).

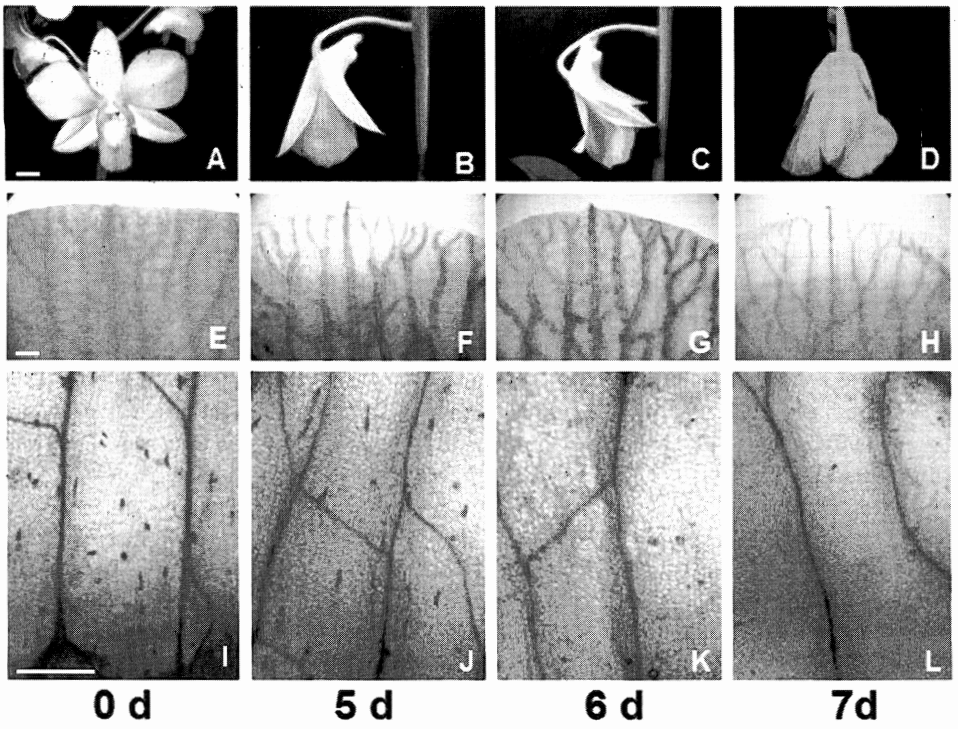

Fig. 2 Visible changes of petal senescence in orchid exposed to ethylene during senescing process after 5, 6 and 7 day. A-D, Appearance of a floret; E-H, Photomicrograph of petals; I-L, Photomicrograph of petals stained by TNBT solution. Scale bars indicate 10 mm (A) and those of (B-D) are omitted; 1 mm (E) and those of (F-H) are omitted; 1 mm (I) and (J-L) are omitted respectively. Pictures arranged lengthways indicate 0, 5, 6 and 7days after ethylene exposure, respectively.

ethylene treated and control plants were taken out every morning from the two growth cabinets, respectively. Mainly the first and second florets of each flower stalk were chosen as the subject of inspection. The state of visible senescence for each floret was evaluated daily based on the degree of both wilting and browning (Fig. 1). Life stages of a floret were defined by the following five degree of wilting: 1 (Fig. 2A), fully opened; 2, turned upside down and drooped (data not shown); 3, slightly wilted whole petal (Fig. 2B); 4, severely wilted whole petal (Fig. 2C); 5, severely wilted and browned whole petal (Fig. 2D).

Measurement of NMR relaxation times (T_1, T_2)

A ^{1}H-NMR spectrometer with a magnet operating at 25 MHz for ^{1}H (Mμ25A, JEOL Ltd., Japan) was used for the measurements of T_1 and T_2. Petal pieces were used for the measurements of NMR relaxation times. Sample was put into an NMR tube (10 mm in diameter) which was then set in the NMR spectrometer. For T_1 measurements, the saturation recovery method (90°-τ-90° pulse sequence) was used (Fig. 6A). For T_1 measurements, the repetition time was between 5 to 8 s, with 4 accumulation transients for each tissue. The decay between scans was always five times greater than T_1. In this method, T_1 is determined from $M_\tau = M_0[1\text{-}\exp(-\tau/T_1)]$, where M_τ is the magnetization amplitude of proton at interval time τ, and M_0 is the magnetization amplitude of proton in the equilibrium state. In this experiment, a free induction decay (FID) signal at every interval time, τ, was obtained by the accumulation of 4 scans. For measurement of T_1, the repetition time of the sequence was always kept more than five times of T_1.

T_2 was measured by the Carr-Purcell-Meiboom-Gill (CPMG) method (Fig. 6B). T_2 is determined from $M_{2n\tau} = M_0 \exp(-2n\tau/T_2)$, where M_0 in the magnetization amplitude of the proton signal occurring at time 2τ after the initial 90° pulse in CPMG (90°x-τ-180°y-2τ-180°y-2τ……) pulse sequence. The T_2s were calculated based on 500 echo signals acquired by accumulation of 16 scans. The solid-echo (90°-τ-90°) method was also applied for measurement of T_2 below 1 ms (Fig. 6B). For this T_2 measurement, repetition time of the pulse sequence was also kept more than five times of T_1. The solid echo signal was obtained by accumulation of 128 scans. $M(t) = \Sigma ai \times \exp[-(t/T_2 i)^{mi}]$ where *mi* is the Weibull coefficient, and *ai* is the signal intensity in each fraction. The relative value of fraction ratio, (*fi*) is calculated by $fi = ai/\Sigma ai$ (Sato, 1994).

A decay curve of echo signal was analyzed by using a non-linear least-square method on semi-log plots of signal intensity (Iwaya-Inoue et al., 2004 references therein). For detailed analysis, two component analysis was carried out. The probe temperature (30°C) was controlled by a thermostat. All data were recorded at this constant temperature and the measurements were conducted with five florets.

Water content

The fresh and dry weight were measured for petals. Dry matter was measured after drying in an oven at 90°C for 20 h. Relative water content was expressed as the ratio of the amount of water to dry matter (g g^{-1} dry matter).

Leakage of electrolytes

After the NMR measurement the petals cut into four pieces (10×10 mm, respectively) were used for measurement of electrolyte leakage. The pieces were immersed in 20 mL of distilled water and shaked at 120 reciprocates min^{-1} for 5 h, and leakage of electrolytes was measured with an electrolyte conductivity meter (Toa conductivity meter, Model CM-20E, Toa Electronics Ltd., Japan) and the extent of leakage of electrolytes was expressed as the percentage of the total electrolytes leaked from each sample, to those from the samples killed by a cycle of freezing and thawing.

TNBT staining

Daily, the edges of fresh petals from the first florets on five spikes were cut into pieces (5×5 mm) and incubated overnight at 25°C in the dark in 50 mM phosphate buffer (pH 7.5) containing 0.1% tetranitro blue tetrazolium (TNBT). Formation of a purplish-black formazan,

accompanied by reduction of the tetrazolium salts by active dehydrogenase (Rosa and Tsou, 1961), was observed with a light microscope (Axiphot, Carl Zeiss, Germany).

RESULTS

Petal senescing processes in orchid plants exposed to ethylene

The several open florets of ethylene-exposed orchid flowers turned upside down and drooped within 12 h (Figs. 1 and 2). The results demonstrated that peduncle-to-flower stalk angle is sensitive to an orchid's reaction against ethylene. Distance between two petals markedly closed at 5 days after ethylene exposure (Figs. 2B and 3). In addition, the orchid petal color entirely changed from pink to brown at 7 d (Fig. 2D, H). In the orchid petals, the control plants retained a constant level of water content at about 11 g H_2O g^{-1} dry weight (92% per fresh wt.) throughout 7 d, whereas the ethylene-exposed clone gradually started to lose their petal water content at 4 d after treatment (Fig. 4). Water contents for the ethylene-exposed florets reached to at 2 g H_2O g^{-1} dry weight (67% fresh wt.) at seventh day after treatment while those in control petals remained constant indicating the initial value throughout the experiment. Control clones maintained a higher degree of water content in the lips as well as petals (data not shown).

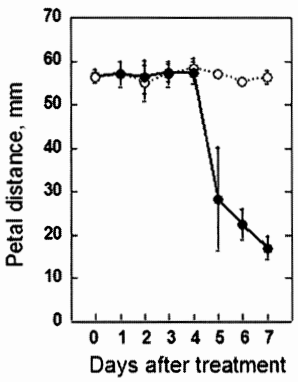

Fig. 3 Changes in distance between petals of orchid plants exposed to ethylene. Control plants (○), Ethylene-exposed plants (●). Symbols represent mean values±standard error (n=5–7).

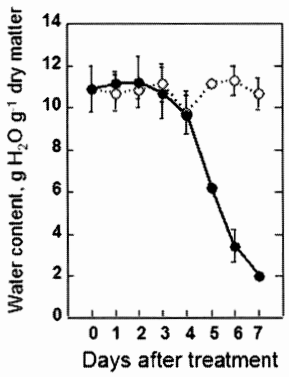

Fig. 4 Changes in water content of the petals of orchid plants exposed to ethylene. Data are indicated as rate of water content per dry matter. Control plants (○), Ethylene-exposed plants (●). Symbols represent mean values±standard error (n=5–7).

Ion leakage markedly increased at 7 days after ethylene exposure (Fig. 5). TNBT staining reflects tissue viability by showing the quantity of active dehydrogenase in existence through observing purplish-black formazan as a result of reduced tetrazolium salts (Rosa and Tsou, 1961; Otsubo and Iwaya-Inoue, 2000). The petal tissues treated by TNBT were well stained in the orchid plants before ethylene exposure, particularly the parenchyma cells adjacent to the vascular bundles (Fig. 2I). After 5 d, nuclei as well as cytoplasm of parenchyma cells of petals in the control clones was still stained (data not shown), but no staining was observed in the ethylene-exposed petals (Fig. 2 J, K, L). TNBT is not reduced to form colored formazan in dead tissues. From these results, it was clearly indicated that flowers of *Dendrobium phalaenopsis* were ethylene-sensitive and exogeneous ethylene regulated a complex physiological changes of petals during seven days.

Water components estimated by NMR relaxation times (T_1 and T_2) in orchid petals exposed to ethylene

T_1 describes the process of realignment of the magnetic moment with the external magnetic field and it becomes a parameter of water mobility. Semi-logarithmic plots of ^{1}H-NMR signal in-

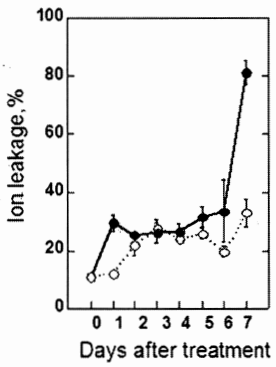

Fig. 5 Changes in ion leakage of the petals of orchid plants exposed to ethylene. Data are indicated as rate of water content per dry matter. Control plants (○), Ethylene-exposed plants (●). Symbols represent mean values±standard error (n=5-7).

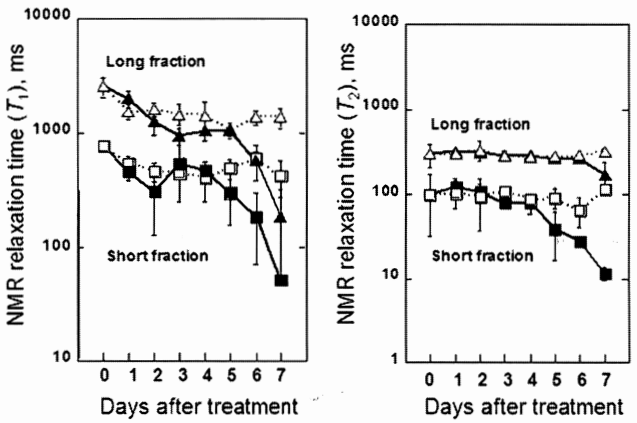

Fig. 6 Changes in NMR relaxation times of the petals of orchid plants exposed to ethylene. (A) Spin-lattice relaxation times (T_1); (B) Spin-spin relaxation times (T_2). T_1 or T_2 values of long fraction of control plants (△), those of ethylene-exposed plants (▲), those of short fraction of control plants (□), those of ethylene-exposed plants (■), Symbols represent mean values±standard error (n=5).

tensity determined using the saturation recovery method were multi-exponential, thus two components of T_1 in the orchid petal tissues during senescing process are shown in Fig. 6A. T_1 values of long fraction in the control orchid clones indicated over 1.0 s, and T_1 values of short fraction ranged between 0.4 and 0.7 s during seven days. T_1 values of the both long and short fractions in the orchid petals were considered as free water derived from intact vacuoles. These results suggested that free water in orchid petal tissues were maintained in the control clones. However, T_1 values of the petals at 5 d after ethylene exposure treatment gradually decreased, indicating that the free water disappeared.

On the other hand, T_2 describes the time-dependent decay of the NMR signal due to the dephasing process of the individual spins with respect to each other. The water component estimated by the T_2 was also divided into two fractions. The water in the petal tissues consisted of at least two water components with T_2 values indicating about 200 to 300 ms and with T_2 values less than 100 ms, respectively (Fig. 6B). Although T_2 values of long fraction maintained the initial values until 5 d after ethylene treatment, fraction ratio markedly decreased at that period (data not shown). T_2 values of the short fraction markedly decreased at 5 d after the ethylene exposure and they reached to 10 ms at 7 d after treatment. The dynamic states of water in the senescing orchid petal tissues exposed to ethylene were closely related to the molecular mobility as indicated by the T_2 values of short fraction than those of the long fraction.

DISCUSSION

Viability phenomena in orchid petals in potted plants exposed to ethylene

Orchids have been proven to be a highly sensitive family to ethylene (Woltering and Van Doorn, 1988). An exhibition of a climacteric-like pattern of ethylene coincided with the first sign of senescence in Dendrobium florets, when the florets showed downward curvature of the ovary (Kesta and Rugkong, 1999). Exogeneous ethylene regulated a complex physiological changes of petals in intact flowering clones of *Dendrobium phalaenopsis* during seven days; decrease in peptal distance (Figs. 1and 2A-D), water content (Fig. 4), NMR relaxation times (T_1s and T_2s) (Fig. 6A, B) and degree of vital staining (Fig. 2I-L), while increase in ion leakage (Fig. 5) and enhancement of translucence like an onionskin (Fig. 2E-H). The change in petal distance is highly likely to have affected the visual decision made on 'degree of wilting' (Figs. 2A-D, 3). Pretreatment of cut florets of a *Cattleya alliances* cultivar with ethylene exposure for 24 h promoted ACC oxidase activity in petals and ethylene production by florets 3 days after harvest, and shortened the vase life (Yamane et al., 2004). Furthermore, it has been suggested that a hyper-phosphorylation status of an unidentified proteins is involved in up-regulating the expression of *Phal-ACS1* gene resulting in increased ethylene production and accelerated the senescence process of *Phalaenopsis* species flower (Wang et al., 2001).

TNBT staining reflects tissue viability. Nuclei of the petal parenchyma cells were clearly observed in a living tissue (Fig. 2I). TNBT staining occurred particularly at the parenchyma cells adjacent to the vascular bundles in control orchid petal tissues (Fig. 2I) while ethylene-treated petals were not stained at 5 d after treatment (Fig. 2J-L). The similar TNBT staining activity has been observed in gladiolus petal tissues treated by trahalose while no staining was observed in control 4 d after harvest (Otsubo and Iwaya-Inoue, 2000). In addition, the orchid petal color changed from pink to brown (Fig. 2E-H), indicating anthocyanin content in petals decreased 7 d after treatment (data not shown). These results indicated that transfer of water from vessels to the petal parenchyma occurred in the control petal tissues while loss in viability was clearly observed in the parenchyma adjacent to the vascular bundles of the ethylene-treated petals.

Ion leakage markedly increased at 7 days after ethylene exposure (Fig. 5). This increase in ion leakage might be due to increased solute permeability of the plasmalemma. It is known that flower

senescence results in enhanced efflux of cellular constituents, such as vacuolar pigments, sugars and electrolytes (Suttle and Kende, 1980; Celikel and van Doorn, 1995), related to loss of turgor and visible wilting (Figs. 1 and 2D, H). The loss of membrane integrity can be modulated by ethylene. An increase in permeability of the tonoplast has been inferred to accompany the increase in plasmalemma permeability in ethylene-insensitive *Tradescantia* as anthocyanins also leak from the cells (Suttle and Kende, 1980).

Vacuolar components estimated by T_1 in orchid petals exposed to ethylene

Dynamic states of water is stated as several water compartment such as free water, loosely bound water and bound water which originate from vacuole, the cytoplasm and the apoplastic region, respectively (Gusta et al., 1979; McCain and Markley 1985; Ishida et al., 1987; Hills and Duce, 1990; Iwaya-Inoue and Nonami, 2003; Iwaya-Inoue et al., 2004). The values of T_1 and T_2 are known to represent different levels of water proton mobility. T_1 values of the long fraction in the control orchid petals were over 1.0 s, while those values of the short fraction were ranging between 0.4 and 0.7 s during seven days (Fig. 6A). Therefore, highly mobile water in the orchid petals was considered to free water derived from intact vacuoles. On the other hand, the T_1 values of both long and short fractions gradually decreased at 5 d after ethylene exposure treatment. Recently, T_1 values of the long fraction in tepal tissues of a tulip cultivar, which are categorized as an ethylene-insensitive plant, showed that trehalose functioned to protect vacuolar water, corresponding to water content (Iwaya-Inoue and Nonami, 2003). In the orchid petals, the control samples retained a constant level of water content at about 11 g H_2O g^{-1} dry weight, throughout the period of storage whereas the ethylene-exposed clone gradually started to lose their petal water content at 4 d after treatment (Fig. 4). The decrease in the water content of the petal tissues during senescence was correlated with T_1 changes (Fig. 6A). The phase behaviors depended on the tissue water content (Belton and Packer, 1974; Iwaya-Inoue and Nonami, 2003).

Furthermore, petal translucence increased, and distance of two petals drastically shorten at 5 d after treatment (Fig. 2B, F). The ethylene exposure to *Dendrobium* sp. induced a formation of abscising layer and leads to abscission of florets, and also prevent water uptake to petals (Fig. 4). These results suggested that transfer of water from vessels to the petal parenchyma was suppressed due to turgor loss, as shown in petal tissues for cut gladiolus flowers (Otsubo and Iwaya-Inoue, 2000; Iwaya-Inoue and Nonami, 2003). Therefore, decrease in cellular water results in the restriction of mobility and metabolites and in the retardation of biological activity (Clegg, 1979).

Bound water estimated by T_2 in orchid petals exposed to ethylene

The water component estimated by the T_2 was also divided into two fractions (Fig. 6B). The water in the control petal tissues consisted of at least two water components with T_2 values of the long fraction indicating about 200 to 300 ms and with T_2 values of less than 100 ms, respectively. These two components have been shown to arise from two distinct water compartments in plant tissues. Difference in T_2 values of biological tissues is interpreted in terms of the differences of the ratio of "free water" to "bound water" (Walter et al., 1989). Regions with T_2 values of the long fraction constitute majority of free water, while regions with the T_2 values of the short fraction constitute of bound water. As shown in Fig. 6B, the fraction with the long T_2 (>100 ms) was mainly associated with vacuole and the fraction with the short T_2 ranging between 10 and 100 ms is thought to be associated with the cytosol and apoplastic region (Chen and Gusta, 1978). Therefore, it is suggested that primary response occurred in relaxation behavior both vacuoles and cytosol of the petal tissues exposed to ethylene. T_2 values of the two components of petals significantly decreased at 5 days after ethylene exposure. Especially, T_2 values of the short fraction in the ethylene-treated petals markedly decreased at 5 d after treatment and reached to 10 ms at 7 d. Therefore, the T_2 values of the short fractions in the orchid petals could be estimated as water fraction with restricted mobility. Abass and Rajashekar (1991) demonstrated a general correspondence between the changes in the major fraction of T_2 for grape stem tissue water, with the lethal

temperature measured by both electrolyte leakage and 2,3,5-triphenyl tetrazolium chloride (TTC) reduction tests. TTC as well as TNBT are not reduced to form colored formazan in dead tissues (Otsubo and Iwaya-Inoue, 2000). A temperature-dependent hysteresis of T_2 revealed irreversible changes in T_2 at the killing temperature. Because the degree of physiological activity in the tissues reflects the level of water binding. From these results, the decrease in the T_2 of the short fraction in the ethylene-exposed plants indicated that viscosity of water in the petals have increased due to exposure to ethylene.

CONCLUSION

Ethylene exposure injured the petal tissues of potted orchid plants. This study indicated basic information concerning NMR relaxation times (T_1, T_2) of water proton in ethylene-exposed orchid flowers. The decrease of NMR relaxation times of the petal senescence was accompanied by decreased vacuolar water, followed by turgor loss, and increased solute permeability. The changes in the vacuolar water and bound water components in the petal tissues could be monitored by T_1 and T_2; the decrease in T_1 and T_2 indicated loss of water mobility, which results in an increase in viscosity, thus decrease in diffusion of substrates and metabolism.

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