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エチレン感受性植物の老化過程における水の物性

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Water Status in an Ethylene-Sensitive Plant during Senescence Processes

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Ethylene can hasten an onset of endogenous rise and senescence in plants. Since ethylene exposure to plants induces a formation of abscising layer and leads to abscission of florets, water uptake to petals from flower stalk might be prevented. We indicated that exogeneous ethylene caused complex physiological changes of petals in intact flowering clones of orchid, *Dendrobium phalaenopsis* cv.during seven days; decrease in both ^{1}H-NMR relaxation times (T_1 and T_2) and water content, and increase in hue angle indicating tone of color with senescence process. Dynamic states of water are stated as several water compartments such as free water, loosely bound water and bound water which originate from vacuole, cytoplasm and apoplastic region, respectively. Long T_1 of the cellular water in the orchid petals decreased and it well corresponded to water content in the ethylene-treated clones. It suggested that vacuolar water disappeared in the petal tissues. Furthermore, long and short T_2s were not maintained in the petal tissues exposed to ethylene. From these results, change in the vacuolar water component of the petals was better monitored by T_1 while the cytosolic water which relates to their molecular mobility could be shown by short T_2 in the orchid petals. This study clearly indicated a basic information concerning petal senescing process evaluated by NMR relaxation times (T_1, T_2) of water proton in the ethylene-sensitive plant.

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[Key words : Flower senescence, orchid (*Dendrobium phalaenopsis*), NMR relaxation times (T_1, T_2), Water compartment ; 花の老化, ラン（デンファレ), NMR緩和時間 (T_1, T_2), 水の成分]

Abbreviations: NMR, nuclear magnetic resonance; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time.

INTRODUCTION

Ethylene is a normal product of plant metabolism and it can hasten an onset of rise and a time to senescence in several fruits and in flowers of some plant families[1, 2]. It is well known that ethylene gas is exhausted by decaying, injured and diseased plant materials. Plants vary widely in their sensitivity to ethylene gas, and orchids have been proven to be a highly sensitive family[3]. Pollinations of orchids lead to localize autocatalytic ethylene production in the stigma and subsequently bring about petal senescence and other post-pollination developmental responses[3]. Characteristically, sexual organs (pistil and stamens) of the orchid flower are fused together into a structure called the column, which lies opposite the lip. A pollen is aggregated together in a number of masses. Orchid flowers are pollinated by a great variety of bees, moths, flies and so on. Therefore, orchid flowers maintain their long lives when they are grown and ornamented in a room. However, applications of ethylene on orchids induce reactions similar to that of pollination and thereby it increased the ethylene-sensitivity[4]. Exogenous ethylene induces premature senescence of orchid flowers when plants are particularly transported with fruits and vegetables stored nearby.

Senescence regulates a complex syndrome of developmental events in many flowers. It is well known that ethylene caused petal abscission in *Pelargonium* cultivars[5, 6]. Additionally, ethylene exposure to *Dendrobium* sp. prevented water uptake to petals[7]. The process of petal senescence was accompanied by decrease of water content, turgor and protein content, and increased ion leakage in gladiolus and cut tulip flowers categorized as non-ethylene-sensitive plants[8, 9].

Since water is the major constituent of tissues in living cells, water status in living tissues is considered to play an important role in the physiological condition in many metabolic processes such as enzymatic reactions, transportation and accumulation of materials occurring in aqueous solution in cells. Nuclear magnetic resonance (NMR) allows determination of changes in dynamic states of water in tissues by detecting relaxation times (T_1, T_2) of water protons, since they reflect the motion of water molecules[10, 11]. Furthermore, 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[12-15]. Furthermore, in petals of gladiolus flower stalk, long T_1 component and short T_2 component are found out to represent free water and loosely bound water, respectively[8]. Although it is known that exposure of *Dendrobium* cut flowers to ethylene induces a set of consequential events[16, 17], there are few study on senescence of potted orchid plants exposed to ethylene.

The objectives of this study were to indicate whether ethylene exposure to potted orchids influenced physical state of water and color change in petals, and to discuss relation to cellular water evaluated by ^{1}H-NMR relaxation times (T_1, T_2) during senescence processes.

MATERIALS AND METHODS

Plant materials

Fig. 1 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. Of the flower's three sepals (outer floral whorls) and three petals (inner floral whorls), all the sepals and the two lateral petals are usually similar to one another in color and shape. The remaining petal, always distinct from them, is called the labellum, or lip ; it is usually larger and different in color and shape, often being lobed or cupped. In this experiment two lateral petals were used as following experiments.

Experimental conditions

Fifty pots of *Dendrobium phalaenopsis* were halved into 2 groups, 25 pots each. Each group was placed in separate growth cabinets. Two growth cabinets indicating

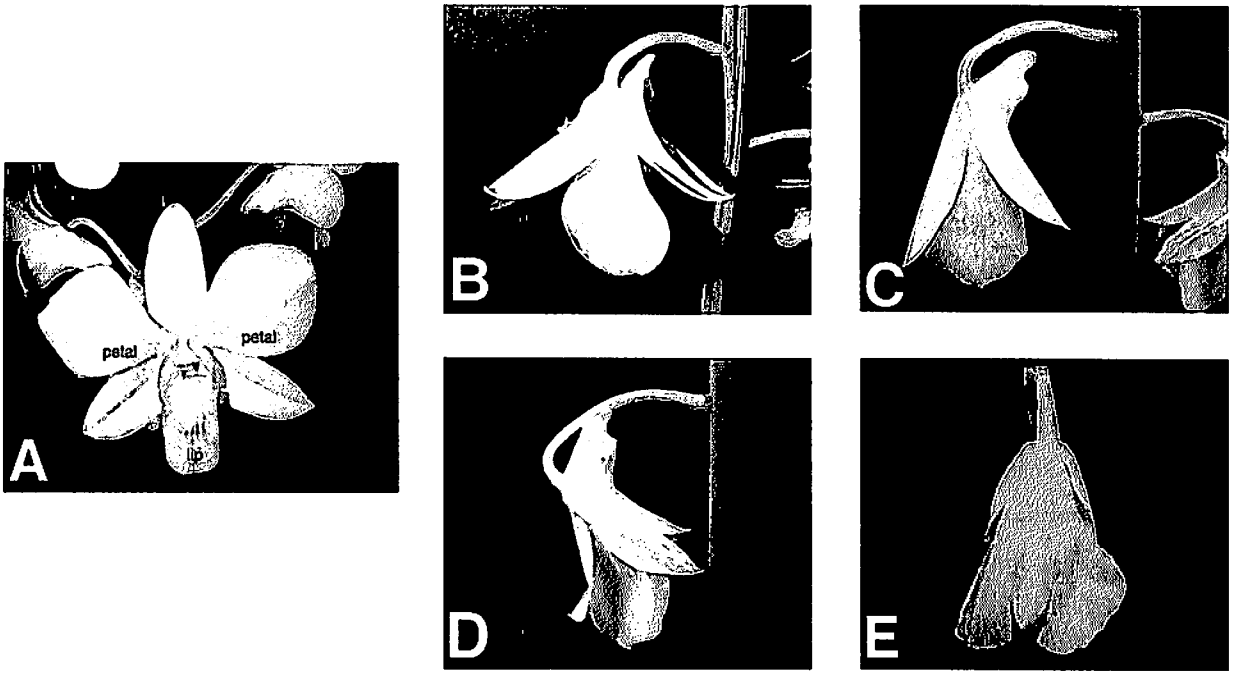

Fig. 1. Visible changes of petal senescence in orchid (*Dendrobium phalaenospis* cv. Linlin) exposed to ethylene during senescing process. The state of senescence for petals was evaluated daily based on the degree of wilting until 7th day. Scores of petal wilting for each floret was evaluated based on the degree of both wilting and browning. Life stages of a floret were defined by the following five degree: (A) fully-opened; (B) fully- opened with pedicel drooping; (C) slightly wilted at the petal edge; (D) slightly wilted whole petal; (E) severely wilted and browned whole petal.

capacity 1.5 m^3 (Koito GC, Koito Mfg. Co., Ltd.) in Kyushu University were each fitted with two gas sampling ports at upper and lower position, respectively. To uniformalize the gas concentration inside, a ventilator was set to assure gas circulation inside. Each growth cabinet was kept at 27℃ and relative humidity of 80 to 90%RH, under 14h of illumination (100 μmol/m^2/s). Ethylene concentrations in the growth cabinets were measured by a gas chromatograph (GC 390 ; GL Science Co. Ltd.), once in 90 minutes after ethylene injection, and once a day in other times. The growth cabinets represented two conditions, ethylene-exposure and control, respectively. The samples were stored in each growth cabinets until the end of experiment.

For ethylene-exposure treatment, on both day 0 and day 1, 50ml of ethylene was applied by introducing it through an injection port. On the other hand, the control plants were remained without ethylene. By gas chromatography, it was ascertained that the flowers in ethylene-exposure were exposed to over 10ppm of ethylene for two to three hours in these two days. The ethylene concentration of the control remained 0 ppm in the whole period of the storage.

Experimental procedures

For the quality evaluations and measurements during their storage period, three pots for the 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. All the scoring and measurements were carried out once a day. The average of the three independent measurements represented the condition.

The state of visible senescence for each floret was evaluated daily based on the degree of both wilting and browning. Life stages of a floret were defined by the following five degree of wilting. Typical examples of these stages are presented in Fig. 1.

Measurement of NMR relaxation times (T_1, T_2)

An ^{1}H-NMR spectrometer with a magnet operating at 25MHz for 1 H (Mμ25; JEOL Ltd.) was used for the

measurement of ^{1}H-NMR relaxation times, T_1 (spin-lattice relaxation time) and T_2 (spin-spin relaxation time). For T_1 measurements, the repetition time was between 5 to 7 s, with 4 accumulation transients for each tissue. The decay between scans was always five times greater than T_1. The values of T_1 were measured based on the saturation recovery method, by a repeated $90^\circ - \tau - 90^\circ$ pulse. The term of τ was the time interval between the two pulses, to the equilibrium state. $M = M_o [1 - \exp(-\tau / T_1)]$, where M and M_o are the proton signal intensity and M_o is the magnetization amplitude of the proton signal.

For T_2 measurements, the repetition time was between 5 to 7 s, with 16 accumulation transients for each tissue. The spectral data of 500 transient acquisitions were accumulated. T_2 were measured by Carr-Purcel-Meiboom-Gill (CPMG) technique. $M_{2n,\tau} = M_o \exp(-2n\tau / T_2)$, where M_o in the magnetization amplitude of the proton signal occurring at time τ after the initial 90° pulse in CPMG ($90^\circ - \tau - 180^\circ - 2\tau \cdots$) pulse sequence.

The both components of T_1 and T_2 were determined by curve fit analysis in semi-log plots of signal intensity of ^{1}H-NMR. Immediately after the sample preparation, initial measurements of T_1 and T_2 were conducted for both samples. The probe temperature (30℃) was controlled by a thermostat connected to the sample chamber of the spectrometer using LN_2. 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℃ for 20h. The relative water content was expressed as the ratio of the amount of water to that of dry matter.

Measurement of petal color

Colors of petals were measured with spectrophotometer (CM-2002; Minolta Camera Co. Ltd., Japan). Using L*a*b* color space, hue angle were calculated. Hue, saturation, and value (HSV) color spaces are often used by artists. The hue is what we normally think of as color. Saturation is the amount of gray, white, or black that is mixed into the color. Zero saturation indicates no hue, just gray scale. The value component of the HSV space is a measure of its brightness. The HSV color space is normalized. Each of its cross sections is a hexagon. At the vertices of each cross section are the colors red, yellow, green, cyan, blue, and magenta. A color in HSV space is specified by stating a hue angle, the saturation level, and the value level. A hue angle of zero is red. The hue angle increases in a counterclockwise direction.

RESULTS AND DISCUSSION

Physiological changes of petals in orchid clones exposed to exogenous ethylene

Ethylene exposure caused complex physiological changes of petals in intact flowering clones of *Dendrobium phalaenopsis* during seven days. The open florets of orchid flowers are shown in Fig. 1A and they turned upside down and drooped at 12h after ethylene exposure (Fig. 1B). At 5 d after treatment wilting markedly increased until 7 d (Fig. 1C, D, E). Hue angle was determined as an index of tone of petal color (Fig. 2). A color is specified by stating a hue angle, a saturation level, and a value level. Petals of the control clones maintained about -25° indicating purplish red color. On the other hand, in the ethylene-exposed plants, hue angle

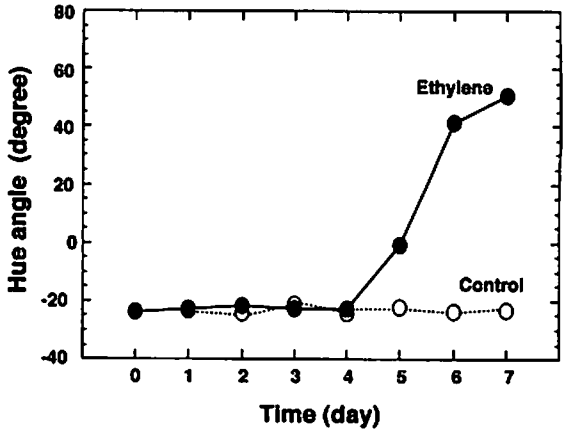

Fig. 2. Hue angle changes of the petal senescence in orchid exposed to ethylene during senescing process. -25° indicates purplish red color. A hue angle of zero is red and that of 180°, as a complementary colors, is cyan. Control plants (○), Ethylene-exposed plants (●). Symbols represent mean values (n = 10 ~ 12).

of the petals increased at 5 d after treatment, and changed the minus value to plus at 6 d. A hue angle of zero is red. Complementary colors are 180° apart and thus angle of 180° is cyan. Eventually, petal color indicated yellow region at 7 d after treatment (Fig. 1E). In addition, the hue angle in leaves exposed to ethylene did not change until 7 d, indicating that ethylene exposure did not affect on leaf color in this experimental procedure. Two petals drastically closed at 5 d after treatment (Fig. 1C). These results coincided with the fact that pedicel-to-lip distance was a sensitive index against ethylene in *Dendrobium* flowers, as reported by Ketsa *et al.*[18].

Water content of the ethylene-treated flowers gradually started to lose at 4 d after treatment and final values indicated about 2 g H_2O/g dry weight while those of control water content maintained at about 11g H_2O/g dry weight until 7 days (Fig. 3). Furthermore, perianth drastically seemed to lose their turgor at 5 d after treatment (Fig. 1C). Ketsa and Rugkong[4] demonstrated that an exhibition of a climacteric-like pattern of ethylene coincided with the first sign of senescence in *Dendrobium* sp. flowers, when the flowers showed downward cuvature of the ovary. As they reported, application of ethylene on cut orchid flowers induced reactions similar to that of pollination. Since ethylene regulates a formation of abscising layer[19], water uptake

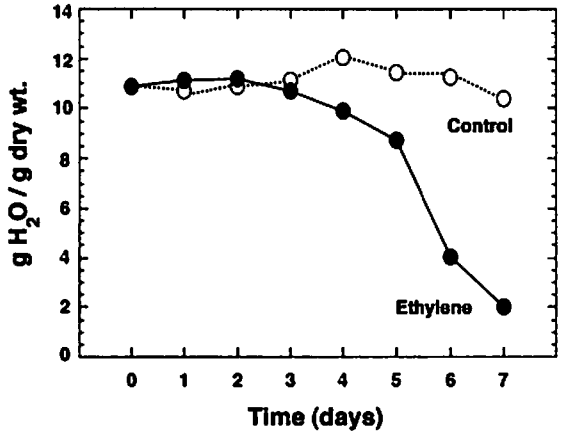

Fig. 3. Water content from the petals of orchid flowers exposed to ethylene during senescing process. Data are indicated as rate of water content per dry matter. Control plants (○), Ethylene-exposed plants (●). Symbols represent mean values (n=5~7).

to petals from flower stalk might be prevent. Therefore, the early response to ethylene suggested that turgor of pedicel tissues could not be sustained. These results suggested that transfer of water from vessels to the petal parenchyma cells for petals was suppressed, as shown in petals for gladiolus spikes[20]. Consequently, turgor loss occurred in tulip tepals[9].

Changes in ^{1}H-NMR relaxation times (T_1 and T_2) in petals of orchid clones exposed to ethylene

Daily changes in NMR relaxation times (T_1, T_2) of the orchid petals exposed to ethylene were shown in Figs. 4 and 5. Spin-lattice relaxation times, (T_1) describes the process of realignment of the magnetic moment with the external magnetic field and it becomes a parameter of water mobility. Normalized T_1 of the petals significantly decreased at 6 days after ethylene exposure (Fig. 4A). Since semi-log plots of ^{1}H-NMR signal intensity were multi-exponential, at least two components of T_1 from water fractions in the orchid petal tissues during senescing process are shown in Fig. 4B, C. Long T_1 values in the control orchid clones were over 1.0s, and short T_1 values ranged between 0.4 and 0.7s during seven days (Fig. 4B). Previous studies indicated that cellular water observed by relaxation times originates from vacuole, the cytoplasm and the apoplastic region[12-15, 21]; highly mobile water is considered to be free water derived from intact vacuoles. In petals of gladiolus flower stalk, long T_1 values ranging 1.2 and 1.8s and short T_1 values ranging between about 0.1 and 0.7s are found out to represent free water and loosely bound water, respectively[8]. Additionally, in fig fruit tissues T_1 values of 1.2s and those ranging between about 0.6 and 1.0s are also found out to represent free water and loosely bound water, respectively[22]. The water in the tissues is considered to increase in a large amount of free water during ripening and T_1 values closely corresponded to cell size. Therefore, highly mobile water in the orchid petals indicating over 1.0s in T_1 value was considered to be free water derived from intact vacuoles. Therefore, it is suggested that free water and loosely bound water in the

orchid petal tissues were maintained in the control clones. However, both long and short T_1 values gradually decreased at 5 d after ethylene exposure treatment (Fig. 4B). Additionally, ratio of the long fraction of T_1 decreased at 6 day after treatment (Fig. 4C). Thus, two water compartments determined by T_1 clearly indicated that decrease in vacuolar water at 5 day after ethyelne exposure compared to one component analysis .

On the other hand, spin-spin relaxation time, (T_2) also indicates water mobility and describes the time-dependent decay of the NMR signal due to the dephasing process of the individual spins with respect to each other (Fig. 5). Normalized T_2 values of the petals significantly decreased at 7 days after ethylene treatment (Fig. 5A). Furthermore, it was revealed that the water in the tissues consisted of at least two water components (Fig. 5B, C). T_2 components with long values indicated about 200 to 300ms and with short values were between 60 and 100msin the control petal tissues, respectively (Fig. 5B). On the other hand, short T_2 values in the ethylene-treated petals markedly decreased at 5 d after treatment. Eventually, the short T_2 values reached to 10ms at 7 d after treatment while long T_2 values of petals decreased to about 200ms at 7 d after treatment. In gladiolus petals, long T_2 values were between about 100 and 300ms, and short T_2 values were ranging between about 10 and 80ms during senescing process[8]. That is, three discrete water

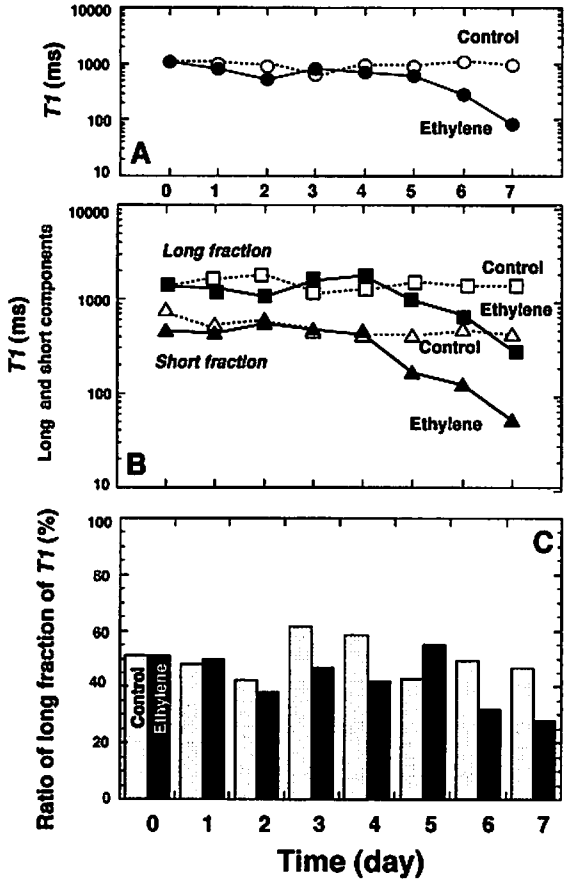

Fig. 4. Spin-lattice relaxation times (T_1) from the petals of orchid flowers exposed to ethylene during senescing process. (A) One component of T_1 values. Control plants (○), Ethylene-exposed plants (●). (B) Two components of T_1 values, long fraction of control plants (□), long fraction of ethylene-exposed plants(■), short fraction of control plants (△) and short fraction of ethylene-exposed plants (▲). (C) Ratio of long component of T_1, control plants are shown by dotted bars and ethylene-exposed plants are shown by closed bars. Symbols represent mean values (n=5).

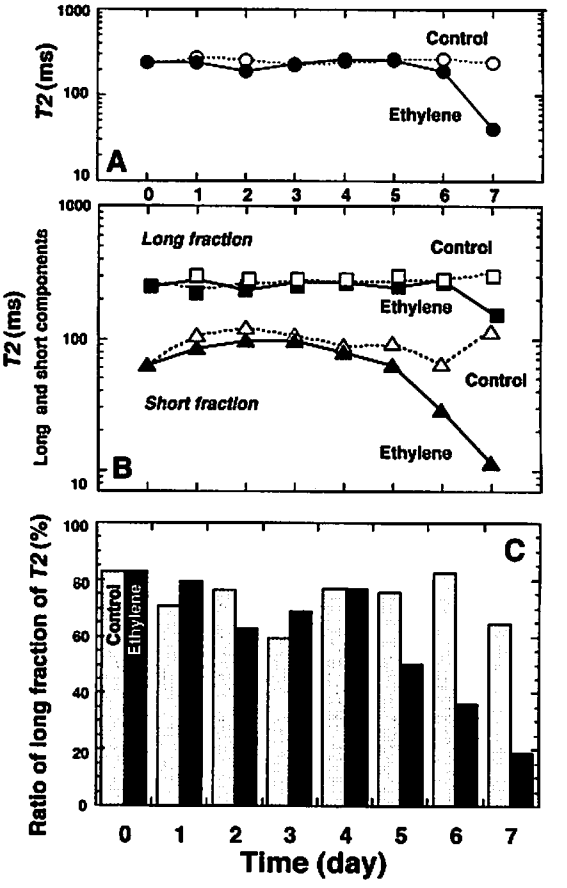

Fig. 5. Spin-spin relaxation times (T_2) from the petals of orchid flowers exposed to ethylene during senescing process. (A) One component of T_2 values. Control plants (○), Ethylene-exposed plants (●). (B) Two components of T_2 values. Symbols are same as in Fig. 4.

compartments in the parenchyma tissues, namely, apoplast, vacuole and cytoplasm where most soluble proteins are contained, had different magnetic environments[23]. Therefore, the long and short T_2 fractions of the orchid petals could be estimated as water fraction with restricted mobility. Although long T_2 maintained the initial values until 5 d after treatment, fraction ratio markedly decreased at that period (Fig. 5C). From these results, ethylene caused shortening of T_1 and T_2 of the orchid petal water with senescing process, and analysis of water compartments clearly indicated that vacuolar water and cytoplasmic water of petals markedly decreased at 5 day after ethylene exposure.

Causative factors affecting water compartments in petals of orchid clones exposed to ethylene

From these results, it was suggested that free water and loosely bound water in the orchid petal tissues were maintained in the control clones. On the other hand, both long and short T_1 values gradually decreased at 5 d after ethylene exposure treatment (Fig. 4B). In the orchid petals, the control samples retained a constant level of water content at about 11g H_2O/g dry weight throughout the period of storage whereas the ethylene-exposed clone gradually started to lose their petal water at 4 d after treatment (Fig. 3). Previous studies have indicated that there are closed correlation with the decrease both in the T_1 values and the water contents for cold acclimated plant tissues[24-26]. Recently, long T_1 of the intracellular water in perianth of gladiolus and tulip, which are categorized as ethylene-insensitive plants, showed that trehalose functioned to protect vacuolar water and prevent water loss due to membrane integrity, corresponding to water content[8, 9]. It has been known that flower senescence results in enhanced efflux of cellular constituents, such as vacuolar pigments, sugars and electrolytes[27, 28], related to loss of turgor. The orchid petal color changed purple to red at 5 days after ethylene exposure (Figs. 1C and 2). Increase in ion leakage might be due to increased solute permeability of the plasmalemma in the petal tissues (data not shown). Loss of membrane integrity can be modulated by ethylene. Therefore, decrease in the long T_1 of the cellular water in the ethylene-treated orchid petals suggested that vacuolar water would not be functioned.

On the contrary, no clear relationship to water content and T_2 determination in gladiolus petals was observed[8]. Also in fig fruit tissues, changes in T_2 corresponded less closely to the water content than those in T_1[22]. When the effects of accumulation of fructose and pectic substances on T_1 or T_2 have been discussed in mature fig fruit, a marked shortening of the T_2 values was observed compared with the T_1 values *in vitro*. It was stated that T_2 reflected compartment size of crosslinked polymer gel[29]. Therefore, it is suggested that T_2 is more strongly affected by the concentration of crystalline water binding site and the thickness of the hydration water multilayer. Since the degree of physiological activity in the tissues reflects the level of water binding, the cytosolic water in the petal tissues exposed to ethylene might be related to their molecular mobility as indicated by short T_2 (Fig. 5). Therefore, decrease in cellular water resulted in the restriction of mobility of metabolites and in the retardation of biological activity.

In conclusion, ethylene exposure caused complex physiological changes of petals in intact flowering clones of *Dendrobium phalaenopsis* during seven days. The processes of the orchid petal senescence were clearly shown by decreased vacuolar water and cytosolic water, and it was accompanied by pigment changes in the ethylene-exposed plants. Change in the vacuolar water component of the petals was better monitored by T_1 while the cytosolic water was related to their molecular mobility as indicated by short T_2.

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