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Root Formation, Root Growth and Root Morphology in Trifoliate Orange Budded with Satsuma Mandarin under Aerated and Stagnant Culture Solutions

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Abstract
The formation rate, elongation and periclinal growths and morphology of roots in trifoliate orange budded with satsuma mandarin, (*Citrus unshiu* Marc, cv. Okitsu Wase) were studied under aerated and stagnant culture solutions. Rooting started simultaneously within a few days of placement of trees to the culture solutions in both conditions, but total roots/tree was higher in aerated solution compared to stagnant one. Incessant elongation growth was found under aerated conditions while growth was slower in the preliminary stage followed by complete cessation after 5 days of induction to the stagnant solution. The aerated solution also attributed gradual root thickening with higher values of stelar and overall diameters and their ratios as well as elongation growth occurred but stagnant conditions inhibited this thickening. Roots at aerated solution had sharply pointed tips in the preliminary stage which remained unchanged even after longer exposure and the epidermal cells were also constantly intact. On the other hand, under stagnant condition, 3-day-induced roots possessed sharply pointed tips which became dome-shaped on the 5th day and variously injured on the 8th and 10th days. Epidermal cells after 8 and 10 days under this condition also became severely injured. This study therefore suggests that satsuma mandarin onto trifoliate orange can endure stagnant condition only 2-3 days but this condition exerts adverse effects on longer exposures.

Introduction
Plants in anoxia have been the subject of experimental studies since the early 1900's considering the fact that higher plants have an absolute requirement for oxygen to sustain metabolism and growth. Oxygen concentration in the root environment relates to plant activities (Willey, 1970; Buwalda *et al*., 1988; Nobel and Palta, 1989; Palta and Nobel, 1989) and lack of oxygen or an excess of CO_2. Both of these conditions can depress root respiration (Jackson and Drew, 1984; Smit and Stachowiak, 1988) which is important for growth, mineral uptake and perhaps, indirectly for water absorption (Lemon, 1962). Oxygen is also indispensable for vital activity in plant roots and that the energy for nutrient and water absorption comes from the oxidation of carbohydrates in roots (Hoagland and Broyer, 1936; Steward, 1935). However, the responses to the changes in aeration have been studied in different crops (Nobel and Palta, 1989; Palta and Nobel, 1989; Zieslin and Snir, 1989; Fox and Kennedy, 1991; Kennedy *et al*., 1992; Zhang *et al*., 1994) using mainly the aboveground parts of plants.

There has long been research interest in the area of other environmental stresses which led to a confluence of research toward new molecular techniques to applied problems and thereby created an increased scientific awareness to environment and stress physiology. Because of this, a tremendous amount of information on the response(s) of plants to various stress, including anoxia has been generated. Yet, the majority of these studies have added only a small segment of anaerobic tolerance in nature to our understanding. Although citrus is not much sensitive to poor aeration, it is fairly intolerable to lack of sufficient oxygen for root respiration and the secondary soil conditions resulting therefrom (Chapman, 1968). A fair share of citrus disorders or substandard performance of stems results from inadequate aeration. Considering the importance of aeration to root health of citrus, this subject has not received a deserving attention and only little is known (Chapman, 1968). This implies that the study of citrus plants under inadequate aeration may provide substantial information on the stress physiology of this plant: Rodts are directly in contact with media environment and are potentially the first line to response. The morphology of roots are also known to be changed along with growth cessation under stress conditions (Mohammad *et al*., 1996). Hence this plant part deserves priority for the studies on growing media-induced effects on the whole plant.

Experiments were designed to elucidate the effects of stagnant culture solution on the formation rate, elongation and periclinal growths and morphological characteristics of roots in trifoliate orange budded with satsuma mandarin compared to forcely aerated solution.

Materials and Methods
Two experiments were conducted under this program in the Citriculture Laboratory, Faculty of Agriculture, Ehime University, Japan during June 22 to August 30 of three consecutive years (1996-1998). Three-year-old satsuma mandarin trees grafted on trifoliate orange rootstocks were utilized. Twelve trees for uniformity of size were placed into six styrofoam boxes containing nutrient solution in each experiment every year. The nutrient solution contained N, Ca, K, S, Mg, P, Na, Cl, Mn, Fe, B, Mo, Zn and Cu at

the following concentration in ppm, respectively: 198, 160, 80, 38, 23, 15, 11, 1, 0.5, 0.4, 0.25, 0.1, 0.05 and 0.02 as was used by Smith (1971). The pH of the culture solutions were adjusted to 5.0 daily by using 1 M H_2SO_4 or 3 M NaOH as required.

In experiment I, the nutrient solutions of three boxes having six trees were continuously aerated and stirred by air pumps whereas another three boxes with six trees were in stagnant conditions. To ensure an adequate supply of all essential elements, the nutrient solutions were completely renewed at 14 days intervals. Regular observations were made on all trees of each treatment and newly formed roots were marked everyday with wax-coated paper tags. The number of roots/tree in different dates for both treatments during the experiment was calculated using these data. The average air temperatures were recorded within 22-29°C during the rooting period.

In experiment II, primarily all the trees were aerated similarly with air pumps. On the formation of numerous new roots, they were measured and were marked with wax-coated paper tags with individual numbers. Fifty percent of the trees were then taken under stagnant condition. The roots after 3, 5, 8 and 10 days of induction were collected and their lengths were again measured. All available roots were measured under this procedure and the elongation growth after specific periods were calculated in both the conditions. For light microscopy, elongated roots were fixed immediately in 4 percent glutaraldehyde, buffered to pH 7.4 in sodium cacodylate, dehydrated in a graded ethanol series, infiltrated and embedded in JB-4 resin. Cross sections of three-micrometer thickness taken at 2, 4, 6, 8 and 10 mm apart from the root apex were stained with toluidine blue 0. The sections were observed and photographed under light microscope. Five roots for each treatment were examined every year. Data on stelar and overall diameters of different distances from the root apex were obtained from the prints. For scanning electron microscopy, roots after 5, 10, 15 and 20 days induction to aeration and stagnant conditions were immediately fixed in 4 percent glutaraldehyde buffered to pH 7.4 in sodium cacodylate buffer, post-fixed in osmium tetroxide, dehydrated in an alcohol series, dried in a critical point drier and coated with gold in an ion sputter. The samples were observed and photographed under HITACHI S-2250N scanning electron microscope at 20 kV.

Results

Formation of roots under forcely aerated and stagnant culture solutions: The inception periods of rooting after placement of trees to the culture solutions were similar under both conditions. Rooting started after 5 days of placement of trees to the culture solutions. Number of roots/tree was higher at aerated condition than that of stagnant solution during the whole period of the experiment every year (Fig. 1). Although rooting varied slightly from year to year, root formation was always higher in aerated solution than that of stagnant one. Root formation was considerably higher from the beginning up to the mid period of the experiment followed by comparatively lower rooting at aerated solution. On the other hand, rooting under stagnant solution was comparable to that of aerated condition in the preliminary stage but subsequently became drastically lower in stagnant solution. The formation of new roots followed a regular trend at aerated condition which means that a considerable number of rooting occurred under this treatment throughout the experimental period. In addition, the drift of culture solution pH at aerated and stagnant conditions also varied considerably. After renewal of culture solution, pH drifts were comparatively higher but subsequently became lower under both situations. in general, the changes of pH was always higher in aerated solution, compared to stagnant one (Fig. 2).

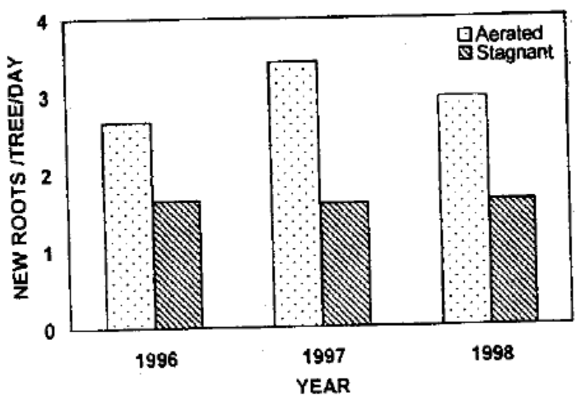

Fig. 1: Number of new root/tree under aerated and stagnant culture solutions during the experiment of 1996-98. The trees under aerated solution produced numerous new roots while rooting was less at stagnant solution

Elongation growth of roots under forcely aerated and stagnant culture solutions: Elongation was higher in aerated condition which continued even up to 10-days of placemen to the treatment as we observed, while growth cessation gradually became distinct under stagnant condition (Fig. 3). Similar results were found in every experimental years. The growth rate always followed a regular trend under aerated condition i.e., the elongation growth continued with superior rates. On the other hand, in stagnant solution elongation growth was comparable to aerated condition at short exposure of 3 days while growth ceassation occured after 5 days of placement.

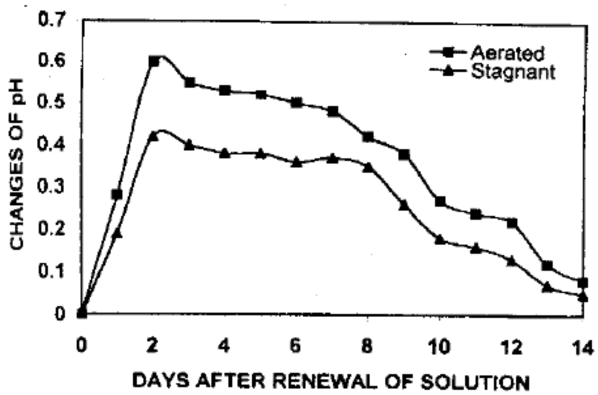

Fig. 2: Gradual changes of pH after renewal of culture solution under aerated and stagnant conditions. The pH drift was up to 0.60 and 0.42, respectively for aerated and stagnant solutions, especially immediately after renewal of culture solution

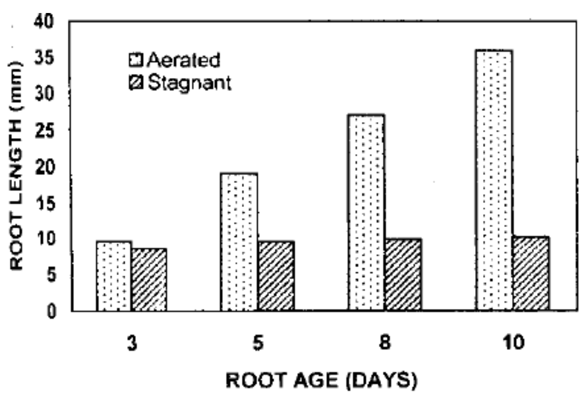

Fig. 3: Elongation growth of roots under aerated and stagnant culture solutions. Rapid and regular growth was found under aerated conditions while growth cessation occurred within 3 days of induction in stagnant solution

Periclinal growth of roots inferred from anatomical records: The periclinal growth in terms of stelar and overall diameters varried considerably in aerated ans stagnant culture solutions. Both stelar and overall root diameters followed a gradual increasing trend toward the base of roots up to 10 mm we studied at aerated condition (Fig. 4, 5). With the increasing of overall root diameter the stelar diameter also increasing and vice-versa under aerated condition. In contrast, the stelar diameter remained similar and overall root diameters followed a fluctuating trend from ratio of stelar to overall diameter also followed the similar terend as stelar and overall diameters under both conditions (Fig. 6).

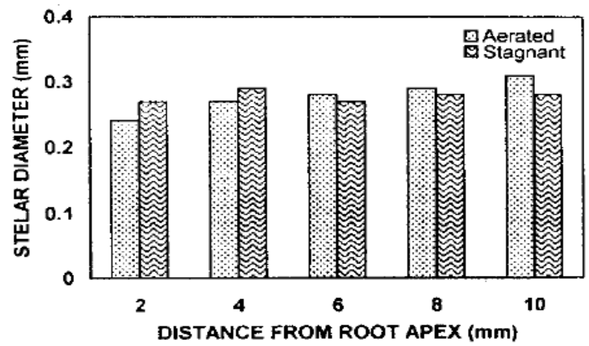

Fig. 4: Stelar diameters of roots of trifoliate orange budded with satsuma mandarin under aerated and stagnant conditions

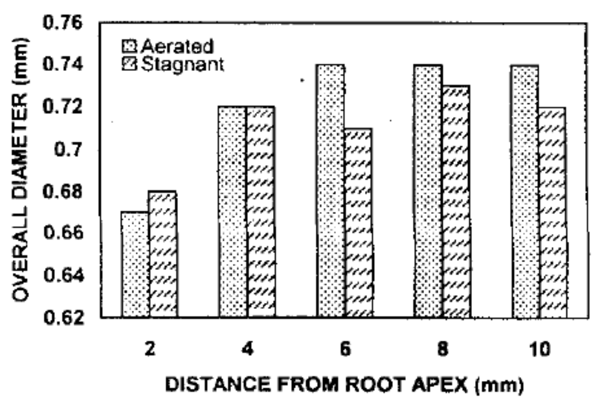

Fig. 5: Overall diameters of roots of trifoliate orange budded with satsuma mandarin under aerated and stagnant conditions

Morphology of roots in aerated and stagnant culture solutions under scanning electron microscope: Roots under aerated culture solution were sharply pointed where normal removel of older rootcap cells were observed on the 3rd day of placement of roots under this treatment (Fig. 7A). The removal of rootcap and older epidermal cells were similarly continued on the 5th day (Fig. 7C). This trend of rootcap removal and epidermal cells continued even up to 8 and 10 days of induction of roots to the condition (Fig. 7E, 7G). In contrast, after 3 days roots had sharply pointed tips under stagnant condition, but severe removals of rootcap and epidermal cells were evident (Fig. 7B). Under stagnant solution roots were comparatively thicker than those of aerated condition at the preliminary stage but the gradual shrinkage of roots in stagnant solution made them narrower and malformed. At stagnant solution, roots were characterized by traces of tip injuries and surface ridging was initiated on the 5th day after placement (Fig. 7D). After 8 days, the whole root surface became vigorously ridged and the tip portion changed to bulbous shape (Fig. 7F). A more severe effects on roots were observed on the 20th day with overall surface ridging and fully damaged root tips (Fig. 7H).

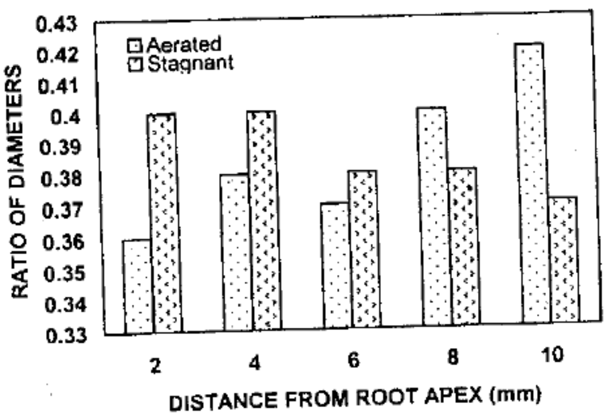

Fig. 6: Ratio of stelar to overall diameters of roots of trifoliate orange budded with satsuma mandarin under aerated and stagnant conditions.

Observations on the root surface exhibited intact epidermal cells in aerated condition. These clear epidermal cells were continuously observed up to 10 days of observations (Fig. 8A, C, E, G). Only the older epidermal cells were found to be removed. In contrast, epidermal cells became deformed or injured under stagnant condition in a short period although the effects of inadequate aeration were not distinct on the 3rd day (Fig. 8B). The cellular damage and epidermal injuries initiated after 5 days of induction (Fig. 8D) which became more conspicuous after 8 days (Fig. 8F). The effects of stagnant conditions became more severe with the complete destruction of epidermal cells on the 10th day (Fig. 8H).

Discussion

Higher number of rooting few days after placement of trees under both treatments and the regular trend of production of roots at aerated condition but less rooting at stagnant solution were conspicuous. This was probably because of the constant favorable physiological state of trees at aerated condition up to the end of the experiment. The comparatively longer induction of trees by stagnant solution exerted adverse effects in a gradual manner. Similarly

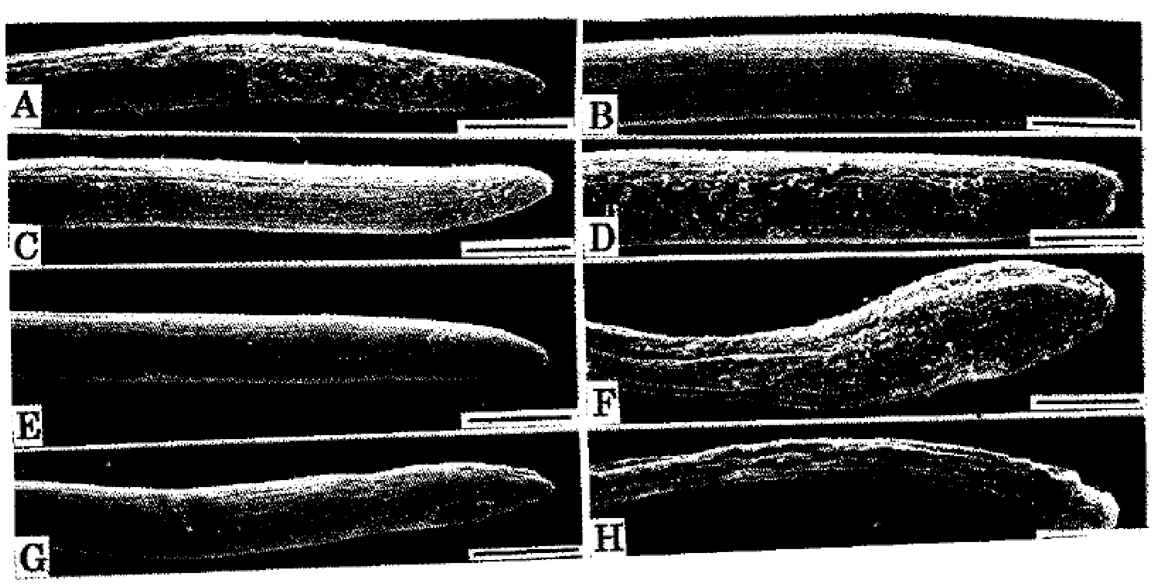

Fig. 7: Morphology of roots of trifoliate orange budded with satsuma mandarin in aerated and stagnant culture solutions under SEM at 20 kV (A, C, E and G represent aerated and B, D, F and H denote stagnant condition). A, B: Three-day induced. C, D: Five-day-induced. E, F: Eight-day-exposed. G, Ten-day-exposed. Note: Continued growth having sharply pointed tips under aerated condition while gradual root tip injuries and surface ridging under stagnant solution. Bar = 1 mm

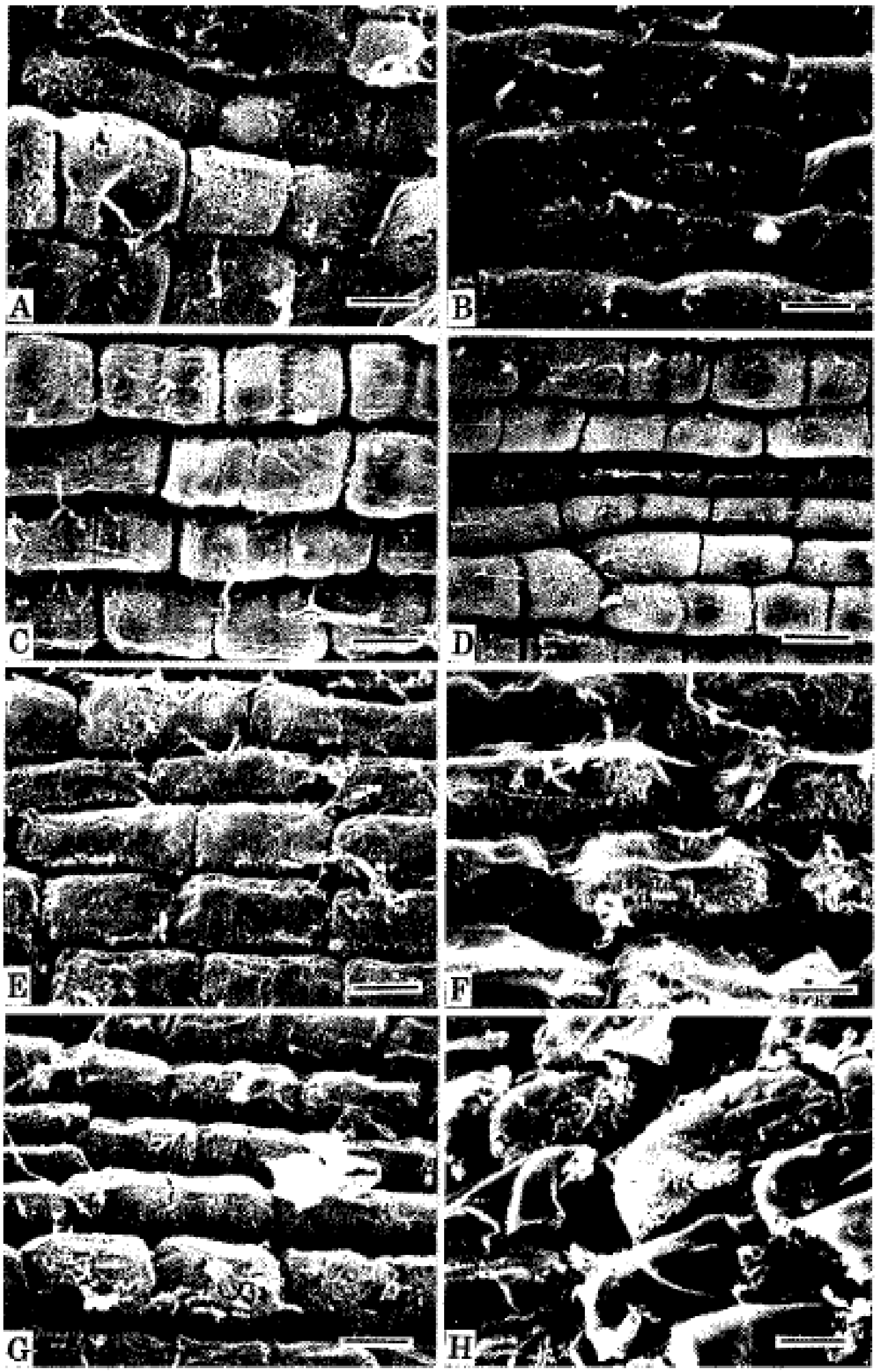

Fig. 8: Localized views of some effects of anaerobiosis on the roots of trifoliate orange budded with satsuma mandarin. A, B: Three-day-induced. Note: The presence of intact epidermal cells. C, D: Five-day-induced. Note: The occurrence of normal epidermal cell under aerated and slightly cellular injuries under stagnant conditions, respectively. E, F: Eight-day-exposed. Note: The distinct appearance of normal epidermal cells under aerated and cellular injuries under stagnant conditions, respectively. G, H: Ten-day-exposed. Note: Evidently intact epidermal cells under aerated and severe cell injuries and death under stagnant conditions, respectively. Bar = 5 μm

Mohammad *et al*. (1996) opined that the adverse effects of low soil moisture and high soil temperature condition on citrus roots were gradual. The present report, however, is adding that not only root growth but the formation of root is also adversely affected by inadequate aeration. The formation of new roots plays an important role to tree vigor and subsequent fruit yield. Therefore, a general remarks can be drawn from this finding that stagnant condition may also exert an indirect influence on the production of this crop through the inhibition of new root formation.

The higher elongation and periclinal growths in plants under aerated condition than the stagnant one conspicuously indicated the inhibiting activities of anaerobiosis to satsuma mandarin plants. Zieslin and Snir (1989) studied rose plants and reported that plant growth in stagnant solution (without forced aeration) was inhibited compared to plants grown in forcely aerated solution. In our study, plant growth was not considered but it can be easily anticipated that if the root growth is checked, there is every possibility of growth inhibition in plant. Therefore, we concurred with Zieslin and Snir (1989). The present study additionally suggests that plant growth inhibition might be caused by the reduction in formation and cessation of growth of roots in the present material under stagnant condition. Higher plants require oxygen for metabolism and the oxygen concentration in the root. environment is directly related to plant activities (Willey, 1970; Buwalda *et al*., 1988; Nobel and Palta, 1989; Palta and Nobel, 1989). Root respiration is required for normal growth of plants (Lemon, 1962). The inhibition of these activities under stagnant solution probably caused root growth cessation. The regular elongation growth up to 10 days of observations under aerated condition and the comparable growth up to the 3rd day followed by complete growth cessation under stagnant solution suggested that trifoliate orange roots can endure the situation for few days followed by serious adverse effects.

Higher changes of pH under both aerated and stagnant conditions immediately after renewal of culture solution following a gradual decrease in fluctuating trend could be understood in our study. However, the causes of higher changes in aerated condition than stagnant condition could be presumed that after changing the culture solution there was a slightly dissolved oxygen which was utilized by plants and the solution became devoid of oxygen in the event of stagnant solution. On the other hand, the state of oxygen concentration was always identical in the aerated solution. Therefore, the higher activities of plants at aerated solutions resulted higher changes of culture solution pH and conversely, the lower activities of plants at stagnant solution brought minor changes.

Although root morphology was normal under stagnant solution in the preliminary stage, subsequently root injuries appeared as an adverse effect. Therefore it can be assumed that tips might be more vulnerable to anaerobiosis than the other portions of roots. As a consequence, the root tip shape became bulbous and injured later. This may also be the steps toward death of roots. The presence of comparatively coarser roots under stagnant solution, in the preliminary stage, may be due to the only development of previously formed cells but reduction in cell division rates in the apical meristem. This assumption, of course, demand ultrastructural level observations of apical meristem cells. The successive narrowing of root shape at stagnant solution obviously indicated the pre-death state of roots. The shrinkage of root surface stood in favor of this opinion. The cell death and injuries in roots under stagnant condition might be due to the lack of respiration resulting slow metabolic activities as have been reported earlier (Willey, 1970; Buwalda *et al*., 1988; Nobel and Palta, 1989; Palta and Nobel, 1989). The present study therefore, indicated that tolerance of trifoliate orange roots budded with satsuma mandarin against inadequate aeration was affected under longer exposure to the condition. However, the complete anaerobiosis could not be ensured in our experiment and the growing state can be termed as stagnant condition. This condition frequently occur in nature due to lack of proper management in the crop field or hardening of the soil crust.

In conclusion, the solution culture of roots in trifoliate orange budded with satsuma mandarin roots under stagnant condition was adversely affected with respect to its growth and morphology. The major effects were reduction in root formation, cessation of both elongation and periclinal growths, cellular death and injuries in the root tip and root surface. Our another study on the effects of anaerobiosis in the same material is going on in the ultrastructural level. That study is expected to be the means of increased understanding of the events in the cell organelle level.

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