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# Changes in Anatomical Features, Pigment Content and Photosynthetic Activity Related to Age of 'Irwin' Mango Leaves

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## Summary

Changes in the anatomical and physiological features of expanding leaves of mango (*Mangifera indica* L., cv. Irwin) were examined to identify the factors that control photosynthetic activity related to leaf age. Anthocyanin content increased before leaf enlargement but decreased rapidly as the lamina expanded. Immature leaves soon after budbreak were yellowish green with a small amount of chlorophyll. When they were stained with 4′,6-diamidino-2-phenylindole (DAPI), some chloroplasts fluoresced red, whereas others were yellowish in the same mesophyll tissues. When the leaf reached almost its maximum area, the chlorophyll content began to increase greatly. With increases in the chlorophyll content of the leaves, the intensity of staining with DAPI increased. Stomata did not differentiate in the leaves soon after budbreak. When leaves reached 28.6 $cm^2$, guard cells of stomata appeared. Green and mature leaves contained higher concentrations of ribulose bisphosphate carboxylase-oxygenase, as detected by immunoblotting after SDS-polyacrylamide gel electrophoresis than did young, immature leaves. The photosynthetic rate and the ratio of variable to maximum chlorophyll fluorescence ($F_v/F_m$) were consistently lower in young, immature leaves than in mature leaves. Oxygen evolution and Fv/Fm ratio increased with an increase of chlorophyll content but Fv/Fm increased much faster during rapid chlorophyll synthesis at the beginning of leaf enlargement than later when the increase in chlorophyll decelerated.

**Key Words**: chlorophyll and anthocyanin, chlorophyll fluorescence (Fv/Fm), 4′, 6-diamidino-2-phenylindole (DAPI), mango leaf, oxygen evolution.

## Introduction

Mango leaves turn from dark brown to green with an increase in area after unfolding. With increases in leaf area, anthocyanin disappears, chlorophyll content increases, and the leaves become thick and rigid. Taylor (1970) described the leaf expansion pattern of mango and the concurrent anatomical changes of mesophyll tissue. Tyagi and Devi (1988) demonstrated that photosynthetic activity is related to chlorophyll content, which depends on leaf thickness. Moreover, light-response curves for the net assimilation rate had a steeper initial slope in leaves that developed in full sun or in 25% shade than those of leaves in 50% and 75% shade (Schaffer and Gaye, 1989a, b). We reported previously that a causal relationship exists between photosynthetic activity and changes in levels of chlorophyll and anthocyanin, as well as between photosynthetic activity and the RuBisCO content, in the leaves of seedling trees from 'Irwin' mango (Nii et al., 1995). However, changes in anatomical and physiological characteristics have not been studied in detail during leaf enlargement and greening in the leaves of 'Irwin' mango. The knowledge on anatomical and physiological changes in the leaves related to leaf age should contribute to our understanding of leaf functions.

A close correlation between $F_v/F_m$ ($F_v=F_m-Fo$) and the optimum quantum yield of the photosynthetic evolution of oxygen has been found in many plant species (Krause and Weis, 1991). The determination of $F_v/F_m$ during the leaf development is important to understand the degree of photoinhibition under natural conditions (Björkman and Demmig, 1987; Castro et al., 1995; Krause et al., 1995). The decline in quantum yield of photosynthesis due to photoinhibition is accompanied by a corresponding decrease in chlorophyll fluorescence (Ögren and Oquist, 1984; Demmig and Björkman, 1987). There are many reports in chlorophyll fluorescence of the leaves in fruit trees and tropical forest trees (Greer and Laing, 1992; Layne and Flore, 1993, 1995; Castro et al., 1995; Krause et al., 1995). Yamada et al. (1996) used chlorophyll fluorescence to show that mature mango leaves are tolerant of high temperatures. Such adaptability of mango may be attributed to their tropical origin where high temperatures and irradiance prevail. Chlorophyll fluorescence is a suitable and rapid indicator in evaluating plant adaptability and photoin-

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hibition (Björkman and Demmig, 1987; Šesták and Šiffel, 1997); however, little is known how the ratio of $F_v/F_m$ depends on changes in leaf pigmentation in 'Irwin' mango trees during leaf development.

Our objectives in studying the mango 'Irwin', were to monitor (1) levels of anthocyanin and chlorophyll, concurrent to the accumulation of starch and ribulose bisphosphate carboxylase-oxygenase (RuBisCO); (2) the differentiation of stomata and mesophyll tissue; and (3) the oxygen evolution and chlorophyll fluorescence ($F_v/F_m$) during leaf enlargement and maturation.

## Materials and Methods

### *Plant materials*

Five 8-year-old 'Irwin', planted in individual boxes (80 liters) filled with sandy soil, were placed in a green-house at Meijo University. The length and width of 5 tagged leaves, approximately 3-5 cm long on non-fruiting current shoots per tree, were measured daily at 9 a.m. until the leaves reached their maximum size. After each measurement, the leaf area was calculated, using the equation $Y=0.426+0.708X$ ($r^2=0.997$), where Y=leaf area ($cm^2$) and X=leaf length × width (cm). The measurements were conducted from May 22 to July 5, 1996. During the experiment, the daily maximum and minimum temperatures in the green-house were 31.9 ± 5.5 ℃ (mean ± SD) and 23.0 ± 2.2 ℃, respectively. Relative humidity changed from 87% to 63%; light intensity at maximum sunlight was 25% less than the outdoors.

The growth velocity and the relative growth rate (RGR) of increase in leaf area were calculated from the following equations: velocity = $(L_2-L_1)/(t_2-t_1)$; and RGR= $(\ln L_2-\ln L_1)/(t_2-t_1)$, where $L_1$ and $L_2$ are leaf areas measured at times $t_1$ and $t_2$, respectively, and $t_2-t_1$ is the number of days.

### *Measurements of anthocyanin and chlorophyll*

During the leaf growth period, leaves were collected periodically for pigment analysis. Levels of anthocyanin and chlorophyll were measured separately on five leaf discs (8 mm in diameter). Anthocyanins were extracted with 10 ml of 1% HCl-methanol and their transmittance of the extract was determined spectrophotometrically at 500 nm. Chlorophyll was extracted with 100% ethanol and quantified by the method of Wintermans and De Mots (1965).

### *Leaf anatomy and observations of chloroplasts stained with DAPI*

For anatomical observations, mature leaves were collected randomly 3 months after budbreak from the middle portion of the shoots and graded into six developmental stages according to leaf color and leaf size (Fig. 1). The central area of the lamina was excised with a razor blade and fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and stored at 4 ℃ until dehydration. Fixed specimens were dehydrated through a graded ethanol series and embedded in Technovit resin (Kulzer, Wehrheim, Germany) for observation under light microscopy. Sections (1.5 μm) were stained with methylene blue and $I_2$-KI solution to follow mesophyll development and starch accumulation, respectively.

To observe the stomata, the central lamina was fixed in 3% glutaraldehyde and 1% osmium tetraoxide and dehydrated through an ethanol series and amylacetate. The specimen was further dehydrated in liquid $CO_2$ coated with gold, and then scanned with an electron microscope.

For chloroplast differentiation and chlorophyll accumulation in the mesophyll cells, palisade and spongy tissues were separated by gentle maceration procedure (Kuroiwa and Suzuki ,1980). After 2 to 3 hr, the tissues were teased apart on a slide and the suspension of cells was observed with a fluorescence microscope. In those chloroplasts exhibiting a face view, the accumulation of chlorophyll was observed with a light microscope using the fluorescent probe 4′,6-diamidino-2-phenylindole (DAPI).

### *Electrophoresis for detection of RuBisCO*

Segments of leaves (0.5 g fresh weight) at similar developmental stages for antomical observation (Fig. 1A) were homogenized in a mortar (chilled with liquid nitrogen) with 5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing of 10 mM ethylenediamine tetraacetate (EDTA), 10% glycerol, 0.2 M NaCl, 4% sodium dodecyl sulfate (SDS), 0.02% antifoam, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, and 5% 2-mercaptoethanol. The electrophoresis were performed as described previously (Nii et al., 1995).

### *Measurements of chlorophyll fluorescence and oxygen evolution*

Chlorophyll fluorescence of 4 to 5 leaves at different developmental stages (Fig. 1A) was measured *in situ* as follows: plants were brought into the laboratory at 8:30 a.m. daily and their leaves were kept in the dark for 30 min to 1 hour at 23-25 ℃ after which the initial and maximum fluorescence emission with Chlorophyll Fluorometer (PAM 101), Actinic Light Control (PAM 102), and Flash Trigger Control (PAM 103) (Heinz Walz, Germany) were recorded. All fluorescence yields were measured from the adaxial surface of the leaf. Fluorescence was excited with light (600-660 nm) from a pulse-emitting diode to obtain $F_0$. Maximum fluorescence yield, $F_m$, was obtained by the application of a 1-sec pulse of saturating light (3,000 μmol $m^{-2}s^{-1}$) to close all reaction centers of photosystem II. Variable fluorescence, $F_v$, was calculated as $F_m-F_0$. The intrinsic or maximal efficiency of photosystem II was calculated as $F_v/F_m$ (Ouzounidou et al., 1995).

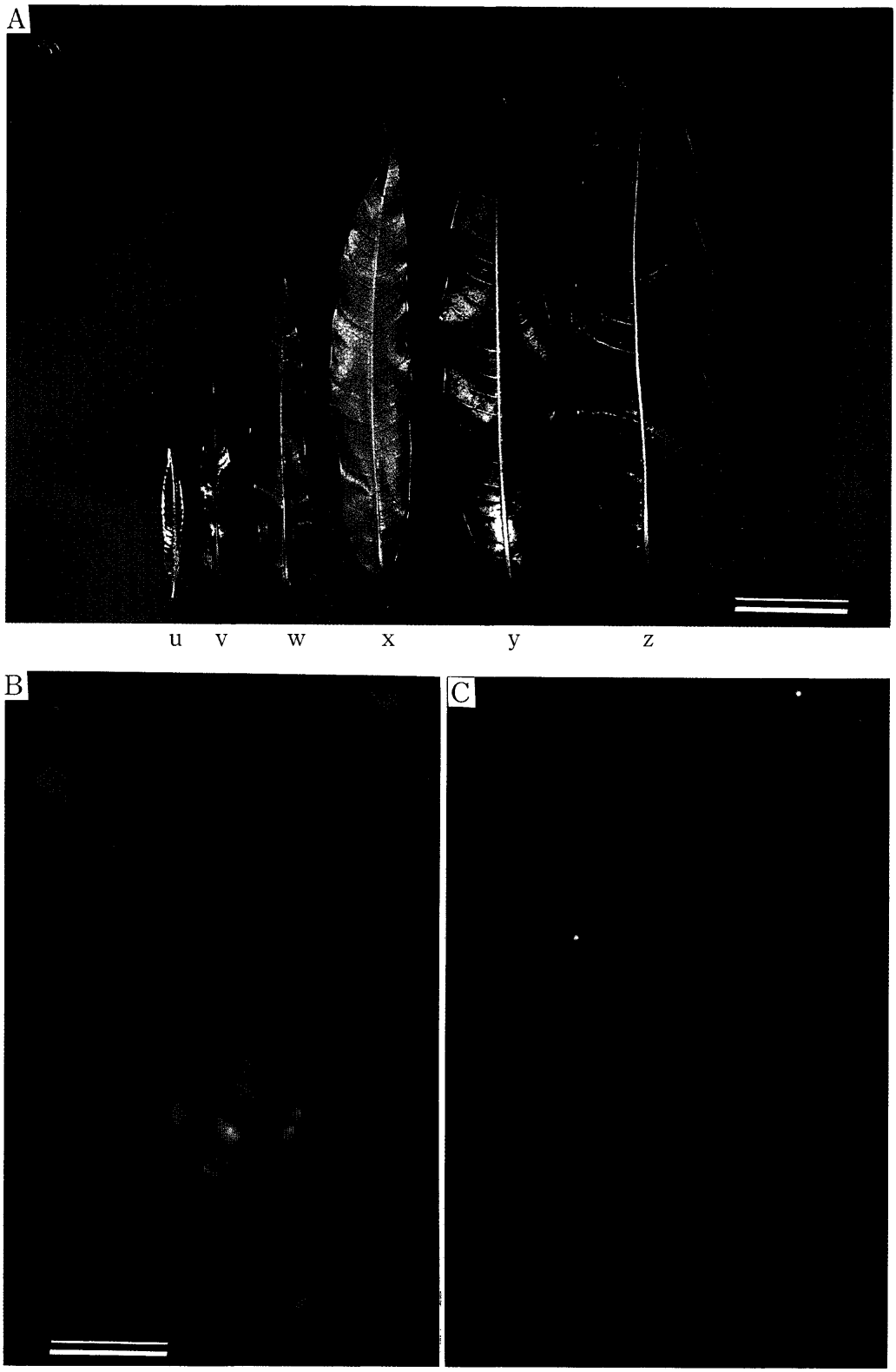


**Fig. 1.** Typical mango leaves illustrating differences in leaf size and color at various stages of development (A). Bar = 5 cm. Fluorescence micrographs showing chlorophyll after staining of mango leaves with DAPI (B and C). Mesophyll tissue was separated by gentle maceration, leaf tissue was teased apart on a slide, and the suspension of cells was examined with a microscope. B, Immature leaf (correspond to v in A) ; C, mature leaf (correspond to y in A) . Bar = 20 $\mu$m.

One day after measurements of chlorophyll fluorescence, the same or similar age leaves were tested for oxygen evolution. Leaf discs (10 $cm^2$), excised with a cork borer, were immediately placed in the chamber and illuminated with actinic light at 30 ℃ under a $CO_2$-enriched atomosphere. The light intensity was 500 $\mu$mol $m^{-2}s^{-1}$ and the wavelength was 600 nm. Oxygen evolution was determined with a Hansatech LD-2 (Norfolk, UK) leaf oxygen-electrode unit, as described by Björkman and Demming (1987).

## Results and Discussion

Immature young leaves soon after budbreak were yellowish or pale green (Fig. 1A-u) but became chocolate-brown (Fig. 1A-v, w). As the leaf enlarged, the chocolate-brown color faded (Fig. 1A-x); when the leaf attained maximal size, the chocolate color disappeared completely (Fig. 1A-y). A mature leaf (Fig. 1A-z), which was collected 3 months after budbreak, was dark green and became very thick and inflexible. When immature young leaves were stained with DAPI, some chloroplasts fluoresced red, whereas others fluoresced yellowish or pale red within the same mesophyll parenchyma cells (Fig. 1B). The chloroplasts in green leaves (Fig. 1A-y) generally fluoresced red (Fig. 1C).

In the very young leaves just after budbreak, the chlorophyll content diminished and anthocyanin increased (Fig. 2A). With the increase in leaf area, anthocyanin disappeared and the chlorophyll content increased concomitantly (Fig. 2A). When leaves approached their maximum size, the chlorophyll content increased rapidly, following a sigmoidal growth curve.

RGR (Fig. 2B) and growth velocity (Fig. 2C) of increase in area of leaves were remarkably high from 4 to 10 days after budbreak. RGR was approximately 0.7 at the initial stage of leaf enlargement, from 4 to 6 days after budbreak, indicating that the leaf area enlarged more than doubled in a day. During the period of high RGR, leaves were very soft and hung vertically. The immature young leaves (Fig. 1A-u, v) after the loss of bud scales were more or less perpendicular to the shoots, but a few days later they rapidly became flaccid (Fig. 1A-w, x). When the leaves had attained almost their maximum size (Fig. 1A-y), they began to turn green, and became rigid and gradually assumed a horizontal orientation. Taylor (1970) reported that for the first 7

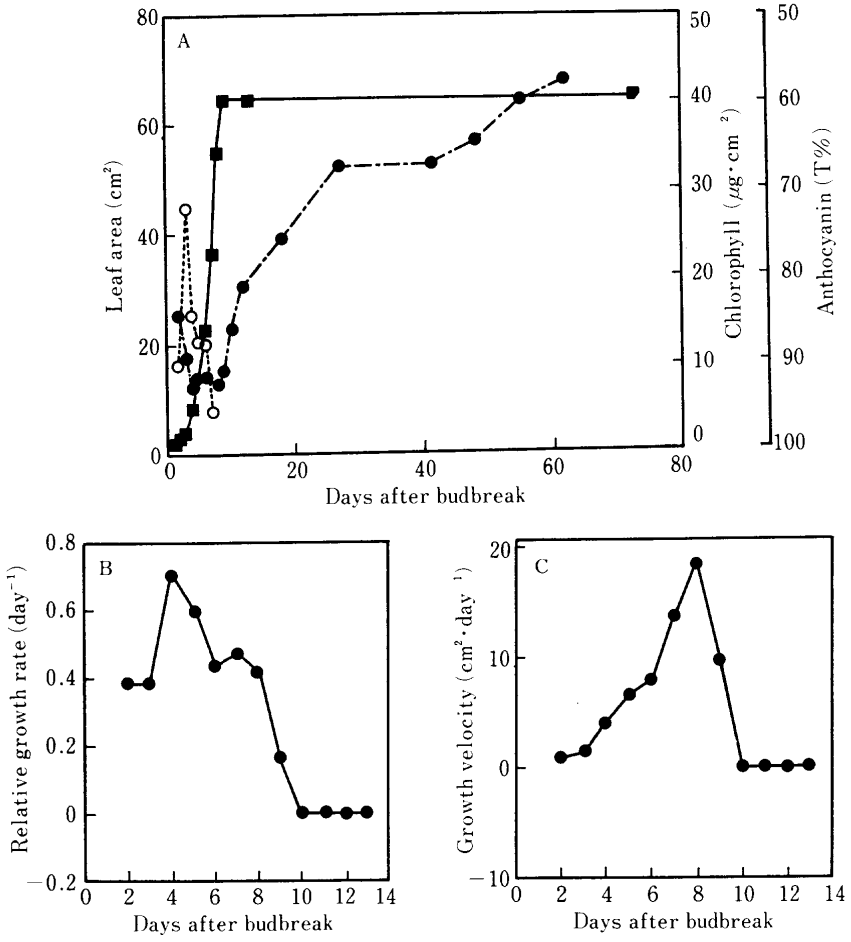


**Fig. 2.** Changes in leaf area (■), and in levels of chlorophyll (●), and anthocyanin (○) (A), relative growth rate (RGR) of leaf area (B), and growth velocity (V) of leaf area (C) after budbreak. RGR = $(\ln L_2 - \ln L_1)/(t_2 - t_1)$ and $V = (L_2 - L_1)/(t_2 - t_1)$, where $L_1$ and $L_2$ are leaf areas measured at times $t_1$ and $t_2$, respectively, and $t_2 - t_1$ is the number of days. Anthocyanin content is the transmittance T% at 500 nm.

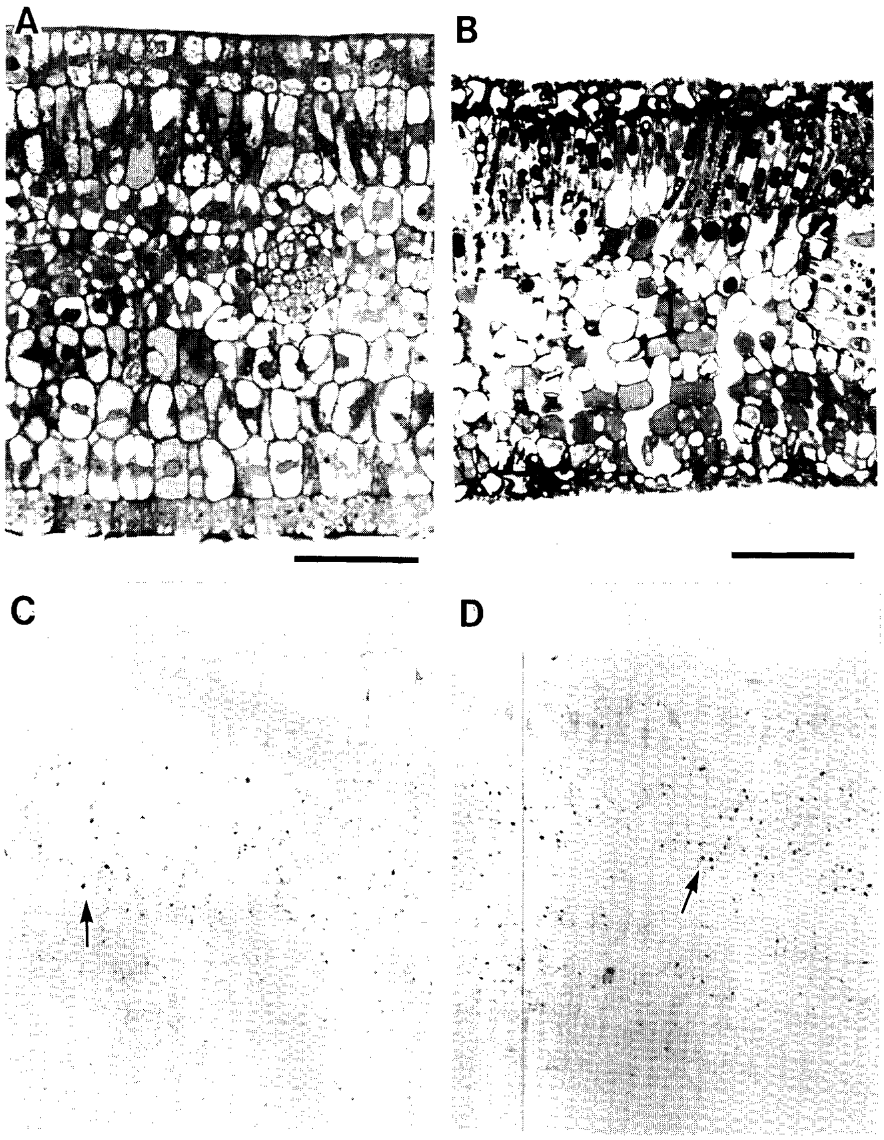


**Fig. 3.** Photomicrographs of cross-sections of mango leaves, showing differences between immature (A and C) and mature green leaves (B and D). A and B stained with methylene blue for observation of leaf and cuticle thickness. C and D stained with $I_2$-KI solution for observation of starch accumulation (arrows point to starch grains). Bar = 50 $\mu$m (A,C), 100 $\mu$m (B, D).

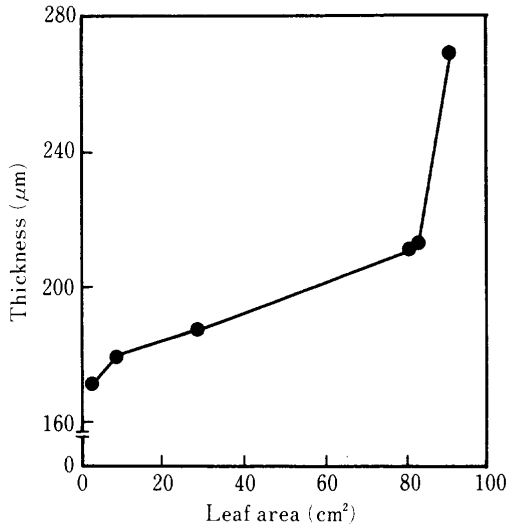


**Fig. 4.** Relationship between lamina thickness and leaf area during 3 weeks after budbreak.

days after the loss of bud scales leaves grew by cell division and by cell enlargement for the next 7 days. Our measurements on 'Irwin' mango confirms that leaf enlargement was rapid for 10 days after budbreak.

In immature young leaves (Fig. 1A-u), the mesophyll tissue had not clearly differentiated into palisade and spongy parenchyma tissues, and the cells were still very compact with little intercellular airspace (Fig. 3A). With increased in leaf area, the palisade cells, which consisted of a single layer of cells, elongated longitudinally. As the leaf fully expanded (Fig. 1A-z), the cuticle became thick (Fig. 3B), and the lamina increased in thickness (Fig. 4). Taylor (1970) described that extension growth of mango leaves was complete 2 weeks after budbreak and that the lamina continued to increase in thickness. He found a linear increase in dry weight which continued for at least the first 6 weeks of growth. Singh and Jha (1939) reported that the maximum rate of photosyn-

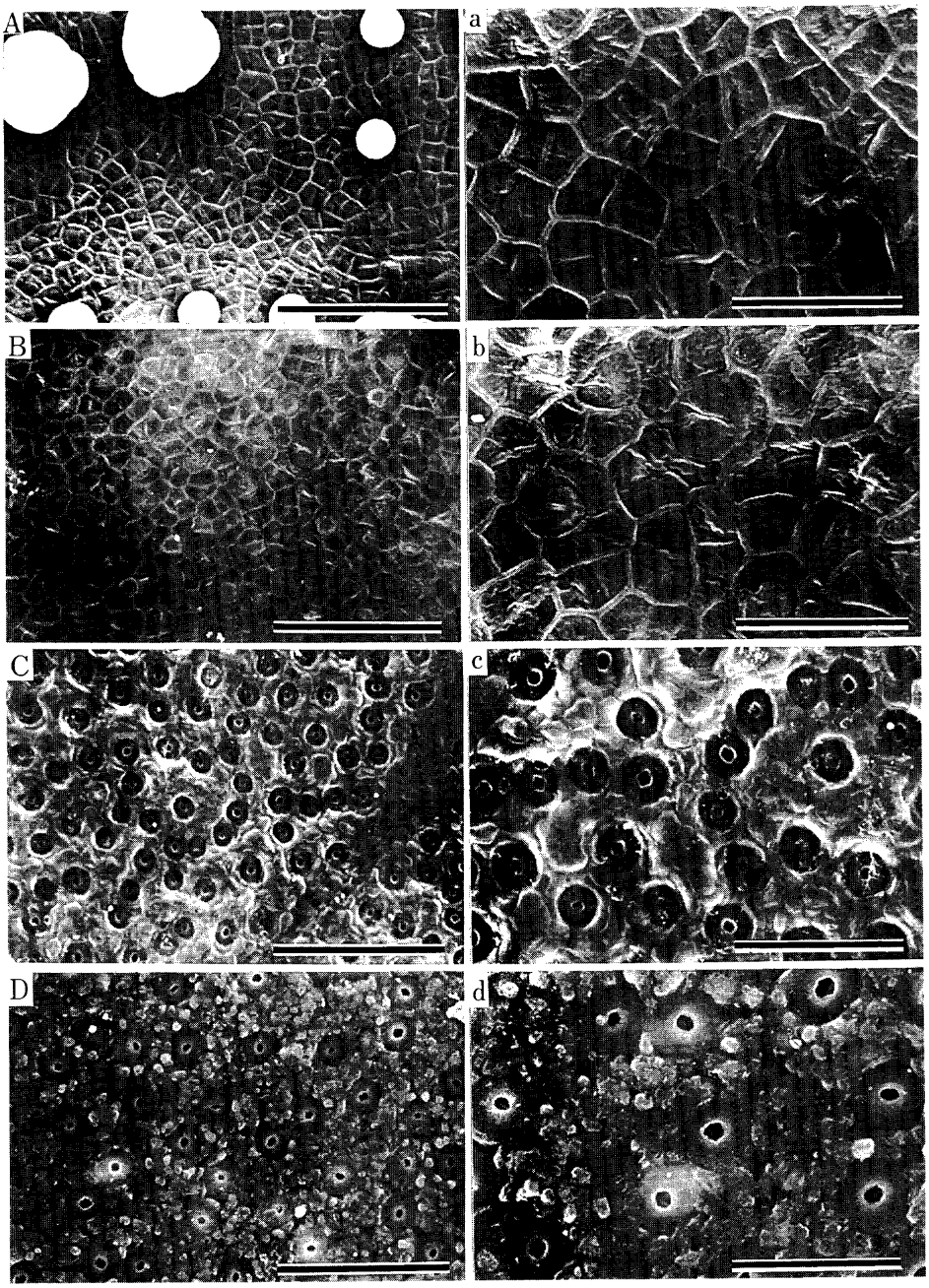


**Fig. 5.** Scanning electron micrographs of stomata on the abaxial side of different age leaves. A, B, C, and D correspond to u, v, x, and z in Fig. 1A ; a, b, c, and d are enlarged parts of A, B, C, and D, respectively. Bar= 100 $\mu$m (C, D), 50 $\mu$m (A, B, c, d), 20 $\mu$m (a, b).

thesis in juvenile mango leaves occurred when they were about 3 weeks old. Hence, mango leaves are most efficient photosynthetically about 2 to 3 weeks after budbreak.

Sections of mesophyll tissue accumulated little starch (Figs. 3C and 3D). Previously, Nii et al. (1995) reported that mature leaves on 8-year-old 'Irwin' seedling accumulated more starch and their chloroplasts were filled with an abundance of starch grains. Although there was a difference in starch accumulation between 'Irwin' and its seedling leaves, our analyses showed that during the greening of mango leaves, the photosynthetic rate was not significantly different between the two (unpublished data). The difference in the starch accumulation in the chloroplasts suggests that photosynthates are exported more readily from leaves of grafted trees.

Stomatal guard cells were not found when the leaf size was 8.8 $cm^2$ (Fig. 5). When the leaf area reached 28.6 $cm^2$, many fully differentiated stomata appeared simultaneously. Although the density of stomata (units per $cm^2$) was high in young leaves, the density of stomata was much lower in mature leaves (Fig. 6).

An electrophoretogram (Fig. 7) of proteins separated by SDS-PAGE shows that the level of L subunit of RuBisCO varied among leaves of different ages. In immature young leaves (Fig. 1A-u, v, w, x), which contained small amounts of chlorophyll, RuBisCO was barely detectable. The level of RuBisCO increased as

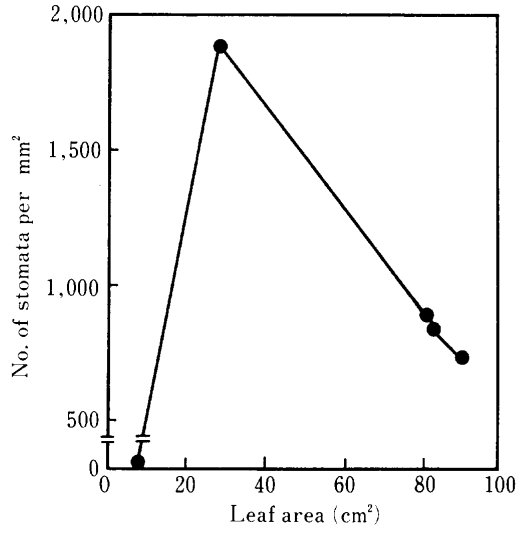


**Fig. 6.** Relationship between the stomatal density and leaf area during 3 weeks after budbreak.

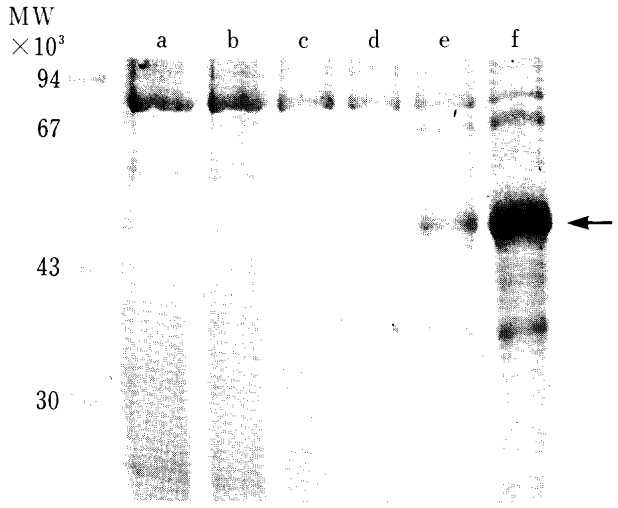


**Fig. 7.** Electrophoretic gel showing the array of RuBisCO during the development of mango leaves. Extracts of leaves were subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue. An equal volume of extracted sample was loaded in each lane (from a to f correspond to u to z in Fig. 1 A). Numbers on the left indicate molecular weights (MW) of pre-stained protein standards. Arrow indicates the L subunits of RuBisCO.

the leaves turned green (Fig. 1A-y). Our results show that the RuBisCO content increased during the greening process after the leaves attained maximum size. Hence, the immunoreactive band in mature leaves (Fig. 1A-z) was markedly dense.

There is a close relationship between the chlorophyll content of leaves and the evolution of oxygen (Fig. 8) and the fluorescence ratio ($F_v/F_m$) (Fig. 9) in 'Irwin' mango leaves. Young, immature leaves had a much slower photosynthetic rate and a lower $F_v/F_m$ ratio than those of mature green leaves. The $F_v/F_m$ ratio increased proportionately to chlorophyll content in younger leaves. The range of $F_v/F_m$ in immature, young leaves was 0.5-

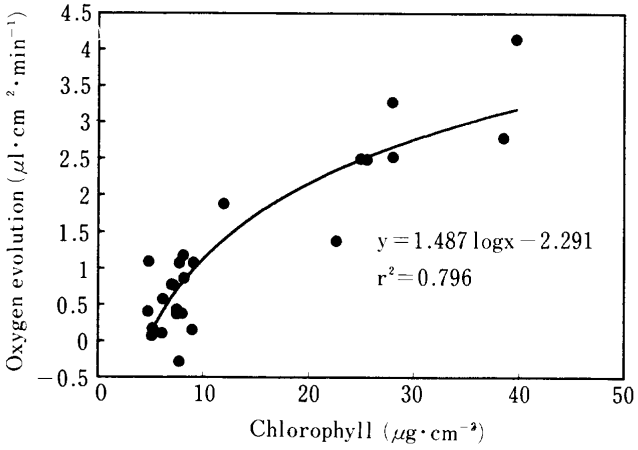


**Fig. 8.** Relationship between oxygen evolution and chlorophyll content of different age leaves.

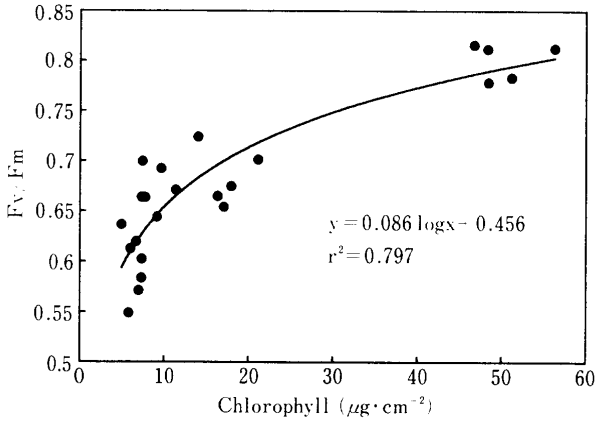


**Fig. 9.** Relationship between chlorophyll fluorescence ratio ($F_v/F_m$) and chlorophyll content of different age leaves.

0.7; the value increased to 0.8 in mature, green leaves. With the further increases in chlorophyll content, its rate of increase slowed down. These results are similar to those described by Björkman and Demmig (1987). .

Björkman and Demmig (1987) suggested that for unknown reasons the $F_v/F_m$ ratio increased initially with an increased chlorophyll content, but further increases in chlorophyll content had little effect on the $F_v/F_m$ ratio. They also described that the slightly lower $F_v/F_m$ ratio of the pale-green leaves may reflect 1) incomplete chloroplast development and 2) chlorophyll content normally found in fully developed green leaves of vascular plants had little effect on the $F_v/F_m$ ratio.

Šesták and Šiffel (1997) noted that chlorophyll fluorescence related to leaf age differed among plant species and that their difference can be larger in some cases than those induced by environmental effects. In *Actinidia deliciosa* $F_v/F_m$ which increased with leaf age was related to increasing leaf diameter (Greer, 1995), whereas in *Gossypium hirsutum* this ratio declined with leaf age (Warner and Burke, 1993). Krause et al. (1995)

illustrated that young leaves responded more sensitively to high light intensity than did mature leaves, as indicated by the pronounced decrease in $F_v/F_m$ ratios and a consistently higher degree of photoinhibition in young leaves.

Immature leaves of mango trees contain a high concentration of anthocyanin but during their enlargement, the anthocyanin content became diluted, while the chlorophyll concentration increased; concurrently, the leaves become thick and rigid. Mohr and Drumm-Herrel (1983) and Dillenburg et al. (1995) explained that considerable amount of anthocyanin in young leaves may form only when necessary as protection from high levels of solar radiation.

The measurements assessed the development of the leaves as functional photosynthetic organs included the leaf area, development of mesophyll tissue, formation of stomata, and leaf chlorophyll concentration. In conclusion, leaves of 'Irwin' mango at immature young stages contained low levels of chlorophyll and RuBisCO content which increased as the leaf expanded; concomitantly, the differentiation of stomata and mesophyll parenchyma cells. The evolution of oxygen and chlorophyll fluorescence ($F_v/F_m$) which were low in young leaves increased markedly with age. This increase is attributed to the combination of increase in leaf thickness (Krause and Weis, 1991) and cuticle development which decreases photoinhibition in mango leaves.

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# マンゴー葉の葉齢に伴う細胞構造，デンプンと色素含量ならびに光合成活性の変化

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## 摘　要

マンゴー‘アーウィン’における葉の光合成機能を解析するための基礎的な資料を得る目的で，葉の肥大生長に伴う形態学的ならびに生理学的変化を調査した．展開直後の葉はクロロフィル含量が低く，黄緑色を呈した．葉が肥大するにつれてクロロフィル含量は一時減少し，その後再び増加した．とくに葉面積が最大に達する時期からのクロロフィルの増加は急激であった．アントシアニン含量は，展開直後は一時的にわずかに増加したが，葉面積の増加とともに急速に減少した．DAPI染色法によって葉緑体を蛍光顕微鏡で観察した．その結果，未成熟葉では細胞の違いによって葉緑体のクロロフィルが赤く励起しているものと黄色に励起しているものなどがみられ，同じ葉肉組織において発達程度の異なる葉緑体がモザイク状態で存在した．葉のクロロフィル含量が増加するにつれて，すべての葉緑体は赤く，強く励起された．気孔は葉の生長初期では未分化の状態であり，葉面積が拡大するに伴って一斉に分化した．葉のデンプン蓄積はすべての葉齢でわずかしか観察されなかった．Ribulose bisphosphate carboxylase oxygenaseをSDS PAGEによる電気泳動法によって比較した結果，未成熟葉に比べて成熟葉で明らかに高かった．葉のクロロフィル蛍光($F_v/F_m$)と単位面積当たりの酸素発生量から葉の光合成能力を検討した．その結果，両者ともに成熟葉に比べて未成熟葉で低かった．葉のクロロフィル含量と酸素発生量との間には高い正の相関関係が認められた．クロロフィル蛍光($F_v/F_m$)はクロロフィル含量が増加するのに対応して増加したが，クロロフィル含量が一定以上の値に達するとクロロフィル蛍光($F_v/F_m$)の上昇はゆるやかになった．