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Identification of Lime Cultivars and Hybrid by Isozyme Patterns

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ABSTRACT

Electrophoretic analysis of leaf extracts was used to identify lime cultivars and the interspecific hybrid of lime and leech lime. Both cold distilled water and phosphate buffer could be used as extraction buffer. In addition, the same isozyme patterns were obtained from the leaf extracts of three-month old cultured seedling and one-year-old potted plants. There was no difference in the peroxidase isozyme profiles in all cultivars of lime and leech lime tested. The esterase isozymes gave better discrimination in cultivars and interspecific hybrid characteristic. The observation of leaf morphology combined with isozyme analyses could differentiate between zygotic and nucellar seedlings after three-month growth in vitro.

Key words : isozymes, electrophoresis, limes, *Citrus aurantifolia*, interspecific hybrid

INTRODUCTION

Common acid lime or lime (*Citrus aurantifolia Swingle*) is a fruit tree belonging to the Rutaceae family. Some native cultivars like Paan', 'Khai' and 'Eman' are very important in current lime production in Thailand. However, these cultivars are very similar in their morphology and difficult to identify until fruiting. The isozyme analysis was used for cultivars characterization of several fruit trees such as mango, kiwifruit and apple (Tansley and Orton, 1983; Weeden and Lamb, 1985; Messina *et al.*, 1991). So this technique may be applied to identify lime cultivars. Moreover, isozyme has been used to distinguish nucellar from zygotic seedlings through the complementation of zymogram profiles between the progenies and their parents (Iglesias *et al.*, 1974; Torres *et al.*, 1982; Anderson *et al.*, 1991).

The objectives of this study were to identify three cultivars of limes and the hybrid between 'Khai' lime and leech lime using polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

The production of interspecific hybrid between lime and leech lime

'Khai' lime (*Citrus aurantifolia*) was hand-pollinated with leech lime (*C.hystrix*) pollen. Hand pollination was carried out immediately following the emasculation of lime flower and the pollinated flower was covered with bags. Two months later the hybrid fruits were harvested and the extracted

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seeds were evaluated for seed number per fruit. After that, seeds were surface sterilized in a solution of 10% Clorox plus 2 drops of Teepol for 10 min, rinsed twice with autoclaved distilled water and germinated on MS medium (Murashige and Skoog, 1962).

Shoot-tip grafting of nucellar and zygotic seedlings

Etiolated 15-day-old pummelo seedlings, grown on MS medium with 75 g/l sucrose were used as rootstock for in vitro micrografting. The pummelo seedlings were decapitated approximately 2 cm above the cotyledons, and the shoot-tips from nucellar and zygotic seedlings were inserted into small vertical incisions made at the cut surface of the stock plants. Successful grafts were transferred into a synthetic soil mix and moved to a plant growth chamber for acclimation before transferred to the greenhouse (Chatisathian *et al.*, 1981).

Leaf samples

The leaf samples of lime cultivars 'Paan', 'Eman', 'Khai' and leech lime were obtained from mature potted plant and seedlings of 3 months old, while a hybrid between 'Khai' lime and leech lime leaves were derived from aseptic grown of 3 months old seedlings. The all samples were washed, wiped dry, weighed (0.5 g) then kept in a deep freezer, prepared for isozyme study.

Extraction

The leaf sample was ground in a mortar, with 2.5 ml of cold distilled water pH 7.0 or 0.05 M phosphate buffer pH 7.5 was added and squashed with a pestle to a fine brei. The brei was transferred into a centrifuge tube and spun at 10,000 rpm for 30 min. The supernatant was collected, mixed with 0.005 % w/v bromophenol blue and 10 % glycerol to show the migration front.

Electrophoresis and gel detection

Polyacrylamide gel electrophoresis was performed in a vertical slab apparatus of discontinuous buffer system with 8.5 % separating gel and 4.5 % stacking gel. The gel buffer was 0.5 M tris-HCl, pH 8.9 and the electrode buffer was 0.04 M tris-glycine, pH 8.3. Amount of 15 and 25 µl of the extracted samples were loaded in each well of the gel for the reaction of enzyme peroxidase and esterase, respectively. A Toyo model ps-1510 with 10 samples/gel was used. The pre-running electrophoresis was necessary conducted at 30 mA in an incubator of 4° C for 10 min. Then the current was increased to 40 mA and maintained for 3 hours.

After the electrophoresis, gels were stained for peroxidase and esterase isozymes as described by Thom and Maretzki (1970). The gels were analyzed for the banding patterns of each enzyme system and photographed.

RESULTS AND DISCUSSIONS

The production of interspecific hybrid between 'Khai' lime and leech lime

Seven hybrid fruits were obtained from 20 pollinated flowers. The average number of seed per fruit and seedlings/seed was 5.28 and 2.63, respectively. There were 71 seedlings obtained in a cross between 'Khai'lime and leech lime which could be classified into two groups. The first group contained 16 seedlings with a winged characteristic intermediate to their parents (Figure 1,2). However, most of seedlings in this group died after growing for 2 months on MS medium (Figure 3). The second group contained 55 seedlings with a winged characteristic similar to their female parent, 'Khai' lime (Figure 1,2). All seedlings were grafted onto the pummelo rootstocks to enhance their growth and vigor. The percentage of successful grafts and plant transferred to soil were 62 and 78, respectively. Twenty-one nucellar and two zygotic plants

survived and were grown in soil for further study.

Peroxidase isozyme

This study demonstrated the same result that using cold distilled water or phosphate buffer pH 7.5 for leaf extraction and the color of peroxidase bands was dark brown all samples. The peroxidase zymograms in 3 cultivars of lime and leech lime were the same. Moreover, the peroxidase zymograms of 3 months old seedlings were also the same as those obtained from mature potted plant. There were two bands of peroxidase which found in two zones; zone I, Rf 0.08-0.09 and zone II, Rf 0.23-0.24. The peroxidase profiles leech lime observed in this study were similar to the reported by Esen and Soost (1976) who used K-acetate; pH 5.4 as extraction buffer. However, it was not possible to identify 3 cultivars of lime and leech lime through the peroxidase isozyme profiles.

For zygotic and nucellar seedlings identification, leaf morphology and the comparison of parental isozyme profiles and 3 months old cultured seedlings were invested. The peroxidase zymograms of seedlings in the first group had 3 bands or 2 bands (Rf 0.03-0.04 and Rf 0.08-0.09) in zone I and 1 band (Rf 0.23-0.24) in zone II. The zymograms of seedlings in the second group had 2 bands, one band (Rf 0.08-0.09) in zone I and the other band (Rf 0.23-0.24) in zone II. It was found that the zymograms of seedlings in the first group differed from their parents. An extra band appeared in zone I, while the peroxidase zymograms of seedlings in the second group were similar to both male and female parents. In this case, it was not possible to determine with certainly the origin of these seedlings using only peroxidase isozymes as marker.

Esterase isozyme

The esterase isozyme presented better descrimination, 12 to 17 bands were detected. The esterase zymograms derived from phosphate buffer pH 7.5 were the same as those produced by using cold distilled water as an extraction buffer (Figure 4). The esterase zymograms of 3 months old seedlings were also the same as those obtained from mature potted plant. The samples showed, the dark gray of esterase isozymes except the extra red-brown band which appeared at Rf 0.50-0.54 in 3 cultivars of lime and leech lime. This band could be seen without staining so it might be used as tracking dye while running the electrophoresis.

The identification of lime cultivars might be made through comparison of major and minor bands. The bands at the cathodal extremity were faint and not consistently scorable, so they were not further discussed. It was found that very similar patterns of esterase were observed in all 3 cultivars of lime tested. Ten bands which appeared in zone I (Rf 0.17-0.46) were the same in all cultivars. The major bands of 'Paan' and 'Eman' lime were located in the range of Rf 0.23-0.29 while these bands in 'Khai' lime were found at Rf 0.27-0.33 (Figure 4). Both 'Paan' and 'Eman' lime were distinguished by the two minor bands in zone II (Rf 0.44-0.46).

To identify the origin of seedlings, leaf morphology and comparison of parental isozyme profiles and 2 months old cultured seedlings were observed. Sixteen seedlings in the first group showed esterase isozyme patterns that included the two bands from the male parent profile, leech lime, and were classified as zygotic characters (Figure 4). The electrophoresis evidence was corroborated by the seedling morphology, since the leaf had winged characteristic similar to their male parent, leech lime. Because of the most of these zygotic seedlings showed strong incompatibility (Figure 3) and died after growing in the aseptic condition for 2 months. Among the hybrid seedling found that only 2 seedlings survived and were grafted onto the pummelo rootstocks (Figure 1). The other 55 seedlings identified, had the same isozyme profiles

Figure 1 Nucellar and zygotic seedlings obtained in a cross between 'Khai' lime (*C. aurantifolia*) and leech lime (*C. hystrix*) were grafteded on pummelo rootstock.

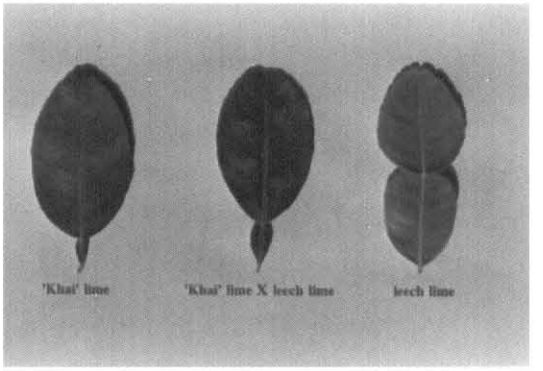

Figure 2 Leaf morphology of the hybrid 'Khai' lime-leech lime plant and its parents ' Khai' lime and leech lime.

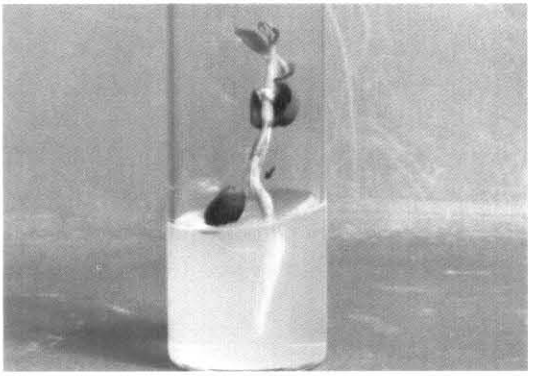

Figure 3 Zygotic seedlings obtained in a cross between 'Khai' lime and leech lime growing on MS medium for 2 months, arrows indicate the eclipse of hypocotyl.

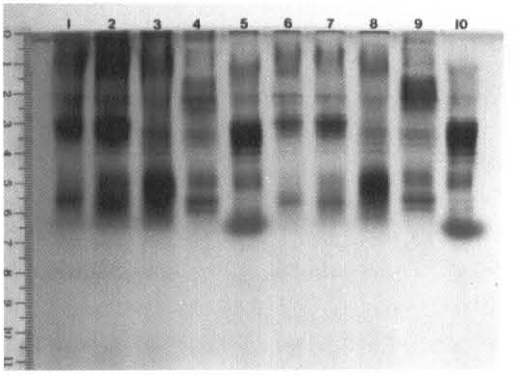

Figure 4 The esterase zymograms derived from leaf of limes, the interspecific hybrid and leech lime extracted with phosphate buffer (lane 1-5) and distilled water (lane 6-10). Arrows indicate two bands characteristic of the male parent, leech lime.

1 and 6 = 'Paan' lime
2 and 7 = 'Eman' lime
3 and 8 = 'Khai' lime
4 and 9 = hybrid of 'Khai' lime and leech lime
5 and 10 = leech lime

as their female parent, 'Khai' lime. So the origin of seedlings in this group was nucellus. Seedlings were also grafted onto the pummelo rootstocks and grown in soil for further study (Figure 1).

CONCLUSION

This study showed that both phosphate buffer and distilled water could be used as extraction buffer for lime leaf extract. In addition, the leaf of 3 months old seedlings gave the same isozyme patterns as one year old leaf of the potted plants. The characterization of 3 cultivars of lime could be resolved through esterase isozymes profiles. 'Khai' lime had the major bands at Rf 0.27-0.33, whereas, 'Paan' and 'Eman' lime had the major bands at Rf 0.23-0.29. These both lime was distinguished with the two minor bands at Rf 0.44-0.46. The peroxidase isozymes showed the poor resolution and the same zymogram of all cultivars. For interspecific hybrid determination, the combination of isozyme analyses and the culture of seedlings in vitro could differentiate the zygotic seedlings after 3 months of growth.

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