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# COTYLEDON DIVERSITY AND SEEDLING CHARACTERISTICS OF *MICHELIA MACCLUREI* DANDY FROM NATURAL POPULATIONS

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## Abstract

*Michelia macclurei* Dandy of natural populations in Guangxi, China had four cotyledon types. In addition to normal dicotyledon (99.2%), three types of cotyledonous mutation (0.8% in total) were observed including monocotyledon (0.1%), tricotyledon (0.4%) and tetracotyledon (0.3%). Cotyledonous mutants were from five of the eight evaluated populations from three of the four counties. In general, unfolding speed of cotyledon and the first true leaf of mutants were slower than normal dicotyledonous seedlings. Compared with dicotyledonous seedlings, monocotyledonous seedling had shorter axial root, fewer number of first-order lateral roots, longer and wider cotyledon. Tricotyledonous seedlings had longer axial root and hypocotyl, higher number of first-order lateral roots, shorter and narrower cotyledons. Tetracotyledonous seedlings had longer axial root, higher number of first-order lateral roots, shorter hypocotyl, shorter and narrower cotyledons. From the study it may be concluded that *M. macclurei* is rich in cotyledon diversity and the morphology of seedlings of cotyledonous mutation differs from normal dicotyledon.

## Introduction

*Michelia macclurei* Dandy is an evergreen and fast-growing tree species of *Magnoliaceae* with high ornamental value (Mai *et al*. 2011) in tropical and subtropical areas, and it is resistant to forest fire (Li and Ruan 1997, Li *et al*. 1998, Lin 2002). Additionally, the wood is excellent for furniture, woodcrafts and building materials (Lian *et al*. 2011, Li *et al*. 2012). However, it is naturally distributed with small forest coverage only in Hainan, Guangdong, and Guangxi provinces of China, and in Vietnam and greatly underutilized (Zheng 1983, Liang *et al*. 2013). The main reason is that the germplasm resource is limited and rarely studied, which significantly restricts tree breeding programs that could potentially maximize the utilization as ornamental or forestry trees.

Cotyledons are the first leaves of a plant and an important part of the seed and embryo. For a given dicotyledonous plant species, the number of cotyledons can vary from two due to gene mutations. Such mutations represent a part of tree germplasm diversity (genetic variation) when occur naturally and are found in many plants (Conner and Agrawal 2005, Liu *et al*. 2008, Korekar *et al*. 2012). Cotyledonous mutants can also be induced manually and have been explored (Chen *et al*. 2006) as certain cotyledonous mutants confer better performance than normal cotyledon formations. For example, tricotyledonous mutants of *Emblica officinalis* Gaertn. and *Cunninghamia lanceolata* benefited the plants by having higher photosynthetic efficiency probably due to larger leaf areas (Pushpkar and Babeley 1990, Yan 2013); apple hybrids with tricotyledonous mutants grew more strongly (Yang *et al.* 1998); tricotyledonous mutants of kiwifruit had thicker trichome covering the leaf surface, which could reduce water transpiration and strengthen self-protection (Zhou *et al*. 2013). Cotyledon diversity has not been reported in *Magnoliaceae* before. Thus an attempt was taken to investigate the cotyledon diversity of eight natural populations of *M. macclurei* in the southern region of China.

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## Materials and Methods

Seeds of *Michelia macclurei* Dandy were collected from 8 natural populations located in Guangxi Zhuang Autonomous Region of China (numbered as 1 to 8). The exact location, forest size and other basic information of each population are presented in Table 1 (Liang *et al*. 2015, Liang *et al*. 2016). Seeds of 2 - 6 randomly selected trees were collected from each site (population). Sample trees are at least 30 m apart. Follicles from each sample tree were kept separately, except for population 8 where seeds from different trees were pooled because follicles were difficult to find. Populations 1 - 6 were collected in early November of 2013, and populations 7 and 8 were collected in late September and October of 2014. The collected follicles were indoor air dried until cracked naturally. The exposed seeds with aril of bright red color were selected and skinned manually, and were properly labeled and stored by mixing with clean medium-coarse sand with 13% water content before testing. A total of 27 seed samples (from 26 trees of populations 1 - 7, and 1 mixed sample of population 8) was included in the study with a total of 2430 seeds (90 seeds each sample) evaluated.

Seeds were germinated following the standard method of the People's Republic of China (GB 2772-1999) of forest tree seed germination evaluation on December 22, 2013 (populations 1 - 6) and December 15, 2014 (populations 7 - 8), respectively. Seeds in groups of 30 were cleaned with tap water, and placed on a layer of absorbent cotton (0.2 cm thickness, as a germination bed) with 2 cm spacing in clear plastic containers (30 cm length × 20 cm width × 12 cm height). The cotton layer was moistened with purified water (Wahaha®, Hangzhou, China) as needed. The containers were placed inside a LRH-250-GSI climate chamber (Shaoguan Taihong Medical Instrument Co., Ltd, Shaoguan, China) at 25°C, 90% RH, and 8 : 16 L : D photoperiod. Observations were made every 1 - 2 days for seed germination. A germinated seed is defined as having the cotyledons fully opened and the first true leaf unfolded (i.e. normal seedling). Each test was terminated when no sign of germination 10 days after the germination peak. Three replications of a total of 90 seeds of each sample were evaluated.

**Table 1. Basic information of the eight natural populations of *Michelia macclurei* in Guangxi, P.R. China.**

| Population ID | County | Longitude (E) | Latitude (N) | Altitude (m) | Forest size (ha) |
|---|---|---|---|---|---|
| 1 | Bobai | 109º37’33.6" | 22º08’49.2" | 110 | 0.9 |
| 2 | Bobai | 109º37’26.6" | 22º08’21.6" | 95 | 1.2 |
| 3 | Bobai | 109º50’26.9" | 21º57’41.7" | 115 | 0.9 |
| 4 | Bobai | 109º50’36.1" | 21º53’24.9" | 210 | 1.4 |
| 5 | Bobai | 109º51’10.1" | 21º54’16.1" | 165 | 2.0 |
| 6 | Pubei | 109º19’39.6" | 22º01’18.1" | 85 | 4.0 |
| 7 | Rongxian | 110º41’4.8" | 21º31’13.6" | 315 | 0.9 |
| 8 | Shangsi | 107º53’22.0" | 21º50’41.0" | 590 | 3.0 |

Seedling measurement was taken for the first 10 germinated seedlings (or all seedlings if less than 10) of each replicate. Seedlings were carefully removed from the germination bed at 10 - 12 days after germination. The length of axial root and hypocotyl, the number of first-order lateral roots, and cotyledon length (the longest point) and width (the widest point) were measured manually. Data of different populations were analyzed by ANOVA followed by Turkey's range test for comparisons between populations.

## Results and Discussion

Overall, 732 (30.1%) seeds were successfully germinated and developed to normal seedlings (Table 2), of which, 726 (99.2%) were normal dicotyledonous seedlings; one (0.1%) was monocotyledonous seedling (Fig. 1a, two additional monocotyledonous seedlings from population 2 failed to develop to normal monocotyledonous seedlings and were excluded from the data); three (0.4%) were tricotyledonous seedlings (Fig.1b); and two (0.3%) were tetracotyledonous seedlings (Fig.1c). Tricotyledonous mutated seedlings have been detected in plants of over 15 families (Conner and Agrawal 2005). In this study, tricotyledony was detected most frequently at 0.4%. This frequency is relatively lower than some evaluated plants, for example, 11.2% in *Ginkgo bilobal* (Li and Qi 1996), 1.05% in *Crotalaria jincea* Linn. (Purkayastha 1940), 0.64% in *Hippophae rhamniodes*, 0.6% in *Brassia oleracea* and 0.53% in *Raphanus raphanistrum* (Korekar *et al*. 2012). But although rare (0.8% in total), cotyledonous mutants of *M. macclurei* were detected in five (population 1, 3, 6, 7 and 2) of the eight evaluated populations from three (Bobai, Pubei and Rongxian) of the four counties, suggesting that cotyledon mutants in *M. macclurei* were occurring in most natural populations. Especially a single sample tree in population 7 had both tricotyledonous and tetracotyledonous seedlings, which accounted for 37.5% of the observed cotyledonous mutants of all populations.

**Table 2. Germination and seedling growth of *Michelia macclurei*.**

| Population ID | Germination (%) | Unfolding of cotyledons (d) | | Unfolding of the first true leaf (d) | |
|---|---|---|---|---|---|
| | | Mean ± SE (n) | Range | Mean ± SE | Range |
| 1 | 55.6 | 32 ± 0.8(99)cd | 21 - 70 | 50 ± 0.9c | 36 - 79 |
| 2 | 18.5 | 38 ± 1.4(100)bc | 19 - 92 | 54 ± 1.4bc | 38 - 104 |
| 3 | 34.8 | 37 ± 1.1(93)bc | 24 - 80 | 49 ± 1.1cd | 33 - 83 |
| 4 | 42.2 | 48 ± 1.1(152)a | 26 - 88 | 66 ± 1.1a | 39 - 99 |
| 5 | 29.6 | 42 ± 1.1(80)ab | 29 - 73 | 60 ± 1.4ab | 41 - 98 |
| 6 | 38.6 | 33 ± 0.8(138)bcd | 14 - 63 | 50 ± 0.8c | 37 - 80 |
| 7 | 11.4 | 38 ± 4.8(38)bc | 18 - 138 | 51 ± 4.7c | 32 - 149 |
| 8 | 28.9 | 27 ± 2.9(26)d | 20 - 97 | 41 ± 3.2d | 28 - 115 |
| Overall average | 30.1 | 38 ± 0.5(726) | | 55 ± 0.6 | |
| Monocotyledon (pop. 3) | - | 39 (1) | | 51 | |
| Tricotyledon (pop. 1, 6 and 7) | - | 42 ± 12.1 (3) | 27 - 66 | 55 ± 8.7 | 41 - 71 |
| Tetracotyledon (pop. 7) | - | 71 (2) | 29 - 112 | 87 | 46 - 128 |

Different letters within a column indicate a very significant difference ($p < 0.001$) in Turkey's test for multiple comparisons; two additional monocotyledonous seedlings from population 2 failed to develop to normal monocotyledonous seedlings excluded in the table; data of unfolding of cotyledons and unfolding of the first true leaf for population 1 - 8 and overall average only include dicotyledons.

Fig. 1. Cotyledon variations of juvenile seedlings of *Michelia macclurei*. a. Monocotyledon seedling; b. Tricotyledon seedling; c. Tetracotyledon seedling.

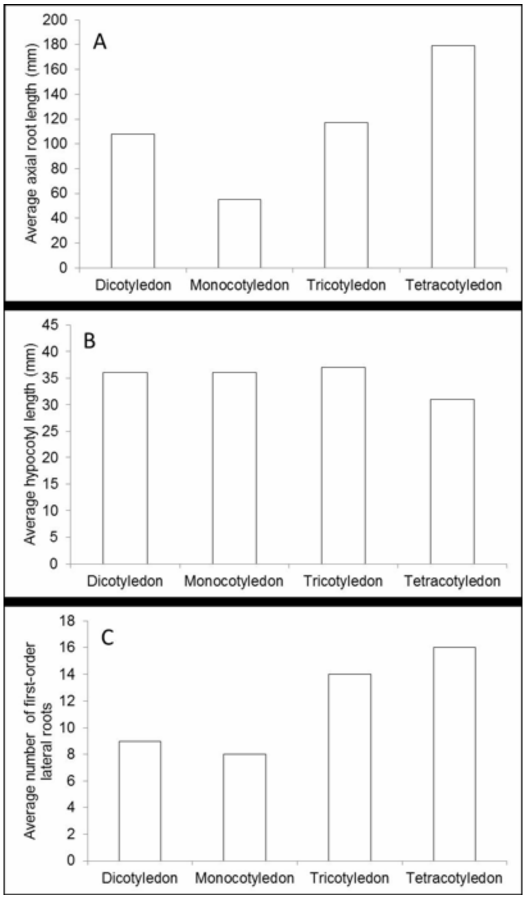

Fig. 2. Juvenile seedling morphology of different cotyledon types of *Michelia macclurei*. A. Average length of axial root (mm); B. Average length of hypocotyl (mm); C. Average number of first-order lateral roots.

When calculated the overall average value of different type cotyledonous juvenile seedlings of 8 populations and compared to the normal dicotyledon seedlings, the axial root of the monocotyledonous seedling was about 50% shorter, while that of the tricotyledonous and tetracotyledonous seedlings was about 8 and 66% longer, respectively (Fig. 2A). The monocotyledonous seedling had similar hypocotyl length with the dicotyledonous seedlings, while tricotyledonous seedlings had longer and tetracotyledonous seedlings had shorter hypocotyl than that of the dicotyledonous seedlings (Fig. 2B). The number of first-order lateral roots of the monocotyledonous seedling (8) was fewer than that of dicotyledon seedlings (9), while that of the tricotyledonous (14) and tetracotyledonous (16) seedlings were more than that of the dicotyledon seedlings (Fig. 2C). Seedlings of *Quercus rubra* L. with more first-order lateral roots had significantly greater survival rate, higher increment of height and diameter at ground level during the first 3 years after field planting (Thompson and Schultz 1995), and rapes with more first-order lateral roots produced significantly more yield (Yang *et al*. 2010). How the tricotyledonous and tetracotyledonous mutated seedlings of *M. macclurei* perform in the field (e.g. grow faster, stronger, etc.) is unknown.

The length of cotyledons of the monocotyledonous seedling was longer, while that of the tricotyledonous and tetracotyledonous seedlings were shorter than that of the dicotyledonous seedlings (Fig. 3). The cotyledon of the monocotyledonous seedling was wider than that of the dicotyledonous type, while that of the tricotyledonous and tetracotyledonous seedlings were narrower (Fig. 3).

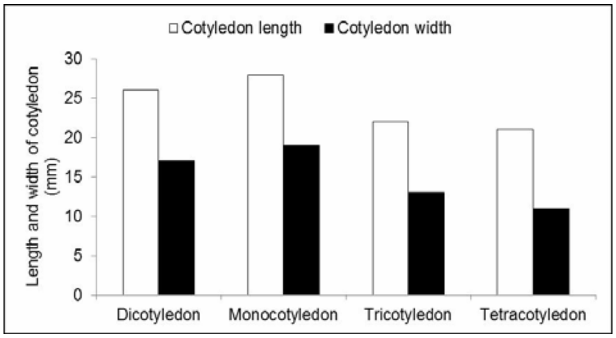

Fig. 3. Cotyledon length and width of different cotyledon types of *Michelia macclurei*.

In terms of the cotyledon unfolding speed ($p < 0.001$) and the first true leaf growth rate ($p < 0.001$), the normal dicotyledonous seedlings were significantly different among populations (Table 2). The monocotyledonous and tetracotyledonous seedlings developed relatively slower and the three tricotyledonous seedlings were faster, slower or similar compared to the normal dicotyledonous type of their perspective populations. This finding is different from a previous report of *Helianthus annuus* L. where tricotyledonous mutant developed faster during seedling establishment stage due to larger leaf areas (Hu *et al*. 2006).

Abnormal cotyledons of dicot plants are results of gene mutations (Conway and Poethig 1997) that may occur naturally or be induced. There are no recorded stressful events near the observed populations of *M. macclurei* such as nuclear radiation, extreme heat or cold, or drought which could induce plants to mutate. The observed cotyledonous mutation of *M. macclurei* most likely caused naturally by fusion (Buchholz 1919), fission, or twinning of cotyledon, and supplementation of the first true leaf (Conner and Agrawal 2005). The rich cotyledonous mutation of *M. macclurei* represents a part of tree genetic diversity, future studies can focus on the advantage and disadvantage of these cotyledon mutants, and how to utilize this genetic diversity to benefit breeding programs and promote *M. macclurei* forest establishment.

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