Scientia Horticulturae 225 (2017) 707–715

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Seed, sugar and acid characteristics of 'Zaomi' Ponkan (*Citrus reticulata* Blanco), a spontaneous mutant of 'Xinnu'

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ARTICLE INFO

Keywords:
'Zaomi' Ponkan
Seed
Male sterility
Self-incompatibility
Sugar
Acid

ABSTRACT

Ponkan is one of the two easy-peeling mandarins grown in China, and 'Xinnu' Ponkan (*Citrus reticulata* Blanco) is a major cultivar in the industry. We selected a spontaneous mutant of 'Xinnu' named 'Zaomi' Ponkan. Compared to the wild type, 'Zaomi' Ponkan exhibited alternating agronomic traits comprising earlier colouring, thinner peel, fewer seeds and higher sugar content. Pollen analysis showed that 'Zaomi' produced a greater number of deformed, nonviable pollen grains. Experiments in self- and cross-pollination suggested that self-incompatibility of 'Zaomi' contributes to the seedlessness of 'Zaomi' × 'Zaomi'. Fruit quality analysis demonstrated that 'Zaomi' was a high-sugar mutant that was not affected by pollinating combination. This study on the fruit characteristics of 'Zaomi' indicates that partial male sterility and self-incompatibility contributes to fewer or no seeds; natural pollination promotes the overall fruit quality of 'Zaomi' Ponkan. These results provide an explanation of the seed formation of 'Zaomi' Ponkan and supply information relevant to its commercial planting.

1. Introduction

Ponkan is one of the two easy-peeling mandarins grown in China; Ponkan produces more than 30% of China's total citrus crop each year. However, sales pressure in the Ponkan industry has increased in recent years as a result of the high yields, the late ripening stage overlapping with that of oranges and a relatively high citrate level immediately after harvesting. Recently we selected a novel Ponkan cultivar named 'Zaomi' that exhibited many excellent traits in fruit seed number and flavour.

Seedlessness is an important aspect of fruit quality. Because of its value both agronomically and economically, the seedlessness trait is sought by citrus breeders and consumers around the world (Deng et al., 1996; Vardi et al., 2008). Satsuma mandarins and navel oranges are successful seedless species that are well accepted by world consumers. At present, several possible mechanisms in citrus seedlessness have been revealed; male sterility is one of the main causes. Classically, seedlessness in satsuma mandarin fruit results from male sterility (Hu et al., 2005). Researchers have also found a similar seedless mechanism in many citrus species such as 'Ougan' (*C. suavissima*) (Hu et al., 2007), Ponkan and 'Nafeng' tangerine (*C. reticulata* Blanco) (Xiao et al., 2007; Yang et al., 2011), 'Huami Wuhegonggan' (*C. sinensis* × *C. reticulata*) (Qin et al., 2015) and 'Meiguicheng' orange (*C. sinensis*) (Huang et al., 2017). Self-incompatibility (SI) is another common mechanism resulting in seedlessness. Past investigators have found sporophyte and gametophyte SI systems, in which the compatibility reaction occurs on stigma and style. In various citrus cultivars, SI is an important mechanism that can produce seedless fruits. Several authors have determined that many pomelo cultivars are self-incompatible from observations of the behaviour of the pollen tube (Yamamoto et al., 2006; Hoang et al., 2014). Researchers have identified many clementine mandarin (*C. reticulata* Blanco) as self-incompatible; the pollen tubes of 40 genotypes grew in the upper or middle part of the style, with none reaching the base of the style (Aka Kacar et al., 2012; Kacar et al., 2015). Moreover, Distefano et al. (2009) found self-incompatible and intercompatible types in some mandarin hybrids. Certainly, seedless citrus fruits could also result from other mechanisms, such as female sterility and parthenocarpy (Yamamoto and Tominaga, 2002), embryo abortion (Chai et al., 2011; Honsho et al., 2015) and polyploidy (Luro et al., 2004; Bosco et al., 2007; Fatima et al., 2010; Grosser and Gmitter,

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http://dx.doi.org/10.1016/j.scienta.2017.08.013
Received 25 April 2017; Received in revised form 8 August 2017; Accepted 8 August 2017
Available online 18 August 2017

2011).

Changes in sugar and/or acid content usually occur in seedless and/or ripening mutants in citrus; both were characteristics of the 'Zaomi' Ponkan mutant in this work. 'Lipeng No. 2' (*C. reticulata* Blanco), a seedless mutant of seedy Ponkan, showed 5.9% and 20% increments in total sugar and acid content, respectively (Cheng et al., 2008). A mutation of Valencia sweet orange exhibited a seedless fruit phenotype as well as higher sugar content and lower acid levels (Luo, 2010). Chen (2010) found a significant decrease in acid level in seedless 'Ougan' relative to its mother cultivar. 'Yanxiwanlu' (*C. reticulata* Blanco), an early-ripening mutant, displayed a slight increase in total sugar content but a marked decrease in total acid level (Xu, 2015). Similar sugar or acid changes also occurred in the 'Fengwan' navel orange, a late-ripening sweet orange (Wu et al., 2014), and 'Late Seedless' mandarin, a late-ripening mutant (Recupero et al., 2008).

In China, 'Xinnu' Ponkan is an excellent native cultivar that originated in Hunan province and has been planted there commercially for more than 30 years. However, the seedy fruit of 'Xinnu' (averaging 11–16 seeds per fruit) reduces its economic value. First found in Xiangxi Autonomous Prefecture, Hunan province, 'Zaomi' Ponkan is a bud sport mutation of 'Xinnu'. The better fruit quality involving fewer seeds, excellent flavour, earlier ripening stage and better fruit shape of 'Zaomi' led to widespread consumer acceptance and recent rapid development in the industry. The new Ponkan cultivar contributes not only to the sustainable development of this industry but also to the study of fruit quality of Ponkan. The present study characterised fruit seedlessness, sugar content and acid levels through natural pollination, self-pollination and cross-pollination during fruit development. The results of this study reveal details about the formation of Ponkan fruit quality and supply important data for commercial planting of 'Zaomi' Ponkan.

2. Materials and methods

2.1. Plant materials

'Xinnu' Ponkan (*Citrus reticulata* Blanco) and its bud mutant, 'Zaomi' Ponkan were investigated in this study. Both cultivars were grown at Xiangxi Autonomous Prefecture, Hunan, China. For characterizing 'Zaomi' and 'Xinu' basically, three relatively uniform trees of each genotype were chosen, and the fruits were harvested at 20, 30, 40, 50, 80, 110, 140, 160, 180, 190, 200, 210 and 220 days after full bloom (DAFB) for analysis. For two cultivars at each sampling point, 10 representative fruits in random collection on the outside crown of each tree were sampled, with a total of 30 fruits per genotype. Endocarps of sampled fruits were isolated, immediately frozen in liquid nitrogen, and stored at −80 °C for further analysis.

2.2. Morphological observation for pollen samples of two cultivars using SEM

Anthers of each cultivar were collected in anthesis. The fresh pollen samples were fixed in FAA immediately. After dehydration using a graded ethanol series (50, 70 and 95%), the samples were subjected to critical-point drying, mounting on copper stubs and sputter coating with gold. The samples were examined under a JSM-6380LV scanning electron microscope (SEM; Jeol, Tokyo, Japan), and representative images were obtained.

2.3. Artificial pollination assay and fruit sampling

To feature the fertilization of 'Zaomi' and 'Xinnu' and its effect on the changes of fruit sugars and acids, another three relatively uniform trees were chosen for each of six pollinating combinations. For cross-pollination, 'Zaomi' and 'Xinnu' were used as female parents of each other with the pollen of the other cultivar. Meanwhile, self-pollination of each cultivar was conducted with flower buds isolation by muslin cloth. Pollination under natural conditions was conducted for each cultivar as control. Each pollinating combination consisted of 800–1000 pollinated flowers. Pollen samples were harvested from freshly opened flowers of male parents. The unopened mature female flowers were emasculated, hand-pollinated, and then bagged and labelled. Four fruits from each tree each pollinating combination were sampled at 150, 160, 190 and 200 DAFB during fruit development. The endocarps were isolated and stored at −80 °C for further analysis.

2.4. Assay of pollen viability and pollen tube growth

Pollen assays were performed following the description of Chai et al. (2011), with slight modification. Ten flower buds from two cultivars were sampled before anthesis; all the pollen samples were freshly harvested. Pollen viability was determined by staining with 1% acetic acid magenta (w/v). One anther per flower bud was collected from six flower buds, mixed and spread on 10 slides (1.2 mm thick).

The styles and ovaries of the hand-pollinated flowers were collected at 1, 2 and 5 days after pollination (DAP). Samples were immersed in a 1:3:1 fixing solution of chloroform, 95% ethanol, glacial acetic acid (v/v/v) for 24 h, then transferred into 70% ethanol, and stored at 4 °C until use. The styles were first washed with distilled water, then incubated in 2 M NaOH for 45 min at 65 °C to soften the tissues, and finally sectioned as thinly as possible. The sections were soaked in 0.1% aniline blue solution for 2–4 h and stained with aniline blue solution. Pollen tubes were observed using a Nikon Eclipse 90i microscope.

2.5. Sugar and organic acid determination

Sugars and organic acids were extracted using the same method used in our previous study (Lu et al., 2016). For each 10 representative fruits from each cultivar, half of the endocarp was homogenized in a homogenizer (MJ-BL25B3, Midea, China) and 3 g of that was used for organic acids and sugars analysis. A 3 g endocarp homogenate was homogenized with 5 mL of double-distilled water and then placed in a water bath at 70 °C, for 30 min. The homogenate was centrifuged at 10,000*g* for 5 min at 25 °C. The residue was extracted twice using the same method; supernatant was collected, and the final volume was adjusted to 25 mL by adding double-distilled water. A sample of 1 mL extract solution was filtered using a 13 mm water syringe filter with 0.22 μm pore size. The filtered solution was used for acid analyses. Acids were analysed by HPLC (LC-20A, Shimadzu, Japan) with an ultraviolet detector (Shimadzu, Japan) operating at 210 nm; soluble sugars were analysed as described by the following method using HPLC with a refractive index detector RI-1530 (Jasco Co., Japan). Triplicate tissue samples were analysed. Sugar and acid components were quantified using the area under the peak relative to that of known standards. All the data were analysed using LC solution software (Shimadzu, Japan).

2.6. Data collection on fruit diameter, seed number, peel colour and peel thickness

For basically characterizing 'Zaomi' and 'Xinnu', we used 30 fruits from three trees to estimate fruit transverse diameter, peel colour and peel thickness during fruit development for each cultivar. Thirty fruits at 220 DAFB were used to estimate seed number per fruit. Fruit transverse diameter was measured around the equatorial plane of each fruit using a vernier caliper, and then the same fruit was cut in half to determine peel thickness around the equatorial plane. Fruit peel colour was determined using a Minolta CR-400 colorimeter with readings at four locations around the equatorial plane. For seed number calculation in pollinating combinations, all the fruits in each combination at 200 DAFB were used. The data were presented as mean ± SD, and mean differences among treatments were evaluated using Duncan's multiple range test in an ANOVA program of SAS (Cary, NC, USA) at $P < 0.05$.