Application of Doyle and Doyle method for DNA isolation from pamelo yellow orange (Citrus maxima Merr), lime orange (C. limon) and sunkist orange (C. sinensis)

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Abstract. Various species of the genus *Citrus* have grown naturally and were cultivated in Indonesia as a mega-biodiversity country. In recent years, the nutritional content and antioxidant activity of citrus fruits has been used to prevent of the various chronic and degenerative human diseases including potential as an antidote to viruses. Development of the potential and superiority of Indonesian oranges including Pomelo yellow orange (*Citrus maxima Merr*), lime orange (*C. limon*) and Sunkist orange (*C. sinensis*) are needed to be done with molecular characterization that begins with DNA isolation. The study was conducted with the aim of applying the Doyle and Doyle method for DNA isolation to all of the three types of oranges. The method for isolate DNA from plant usually use *Cethyl Trimethyl Ammonium Bromide* (CTAB) and *Sodium Dodecyl Sulphate* (SDS). The aim of this study is to isolate DNA from the orange leaves of Pomelo Yellow Orange, Lime Orange and Sunkist Orange with the Doyle & Doyle method. The results of the isolation of orange DNA were measured using NanoDrop. The results showed that the DNA of the three oranges had purity of pomelo yellow oranges 1,83, limes 1.8 and sunkist oranges 1,51. The concentrations obtained from each of the oranges were Yellow Bali Citrus 532 ng / µl, Lime Citrus 664.5 ng / µl and Sunkist Orange 888.6 ng / µl.

1. Introduction

Citrus (*Citrus* sp.) is one of the genera from the Rutaceae family which has the highest economic value. The genetic diversity of oranges is very high, this is indicated by the high number of taxonomies (species and hybrids) [1,2]. *Citrus* itself originates from Southeast Asia and was cultivated in China in 2500 BC [3], where it is referred to as the 'Chinese' apple [4]. Recently, oranges are grown almost all over the world as a food source for humans of high nutritional value, a source of vitamins and other uses. The annual global production of citrus fruit has seen strong and rapid growth in recent decades. Although many citrus fruits, such as oranges, tangerines, and grapefruit can be eaten fresh, about one-third of citrus fruits worldwide are used after processing, and orange juice production accounts for nearly 85% of total processed consumption [5]. Because of their preferred taste, great

taste, affordable economic reach, and growing consumer awareness of their potential health benefits, citrus fruit and products are very prevalent with widespread nutritional and economic impacts in both developed and developing countries [1].

Oranges are one type of fruit that is rich in nutrients and minerals, especially as a high source of vitamin C so it is important for health and also has antioxidants that can help protect cells in the body from free radical damage. The quality of citrus fruit itself can be determined from the level of purity and concentration levels of other compounds contained in oranges. For every 100 g of oranges there are 0.9 g protein, 0.2 g fat, 11.3 g carbohydrates, 23 mg phosphorus, 33 mg calcium, 0.4 mg iron, 190 IU vitamin A, 0.08 mg vitamin B1, 49 mg of vitamin C, and 87.2 g of water. Citrus fruits (*Citrus* sp) have bioactive content in the form of vitamin C, folic acid, carotenoids (especially β-carotene), flavonoids, limonoids, and dietary fiber. Sixteen of the carotenoids that are active as sources of vitamin A are found in citrus fruits [6–9].

Propagation by seed is associated with problems such as poor pollen production, environmental incompatibility and embryonic vigor [10,11]. Therefore, planting onto suitable rootstocks is common to ensure good quality reproduction of known cultures. The use of tissue culture to produce plantlets of good quality, but very susceptible to root disease, and this limits production [12]. Application of molecular technology would increase and facilitate the improvement of Citrus plant. This aplication will start with genomic DNA isolation, which is difficult to perform due to high concentration of polysaccharides polyphenol, proteins, RNA, and secondary metabolite compounds inside them[13]. Good quality and quantity of DNA is needed for various activities such as the use of molecular markers, making genome libraries, to sequencing [14]

There are many methods of plant DNA isolation, such as the use of phenol, CTAB and SDS in DNA extraction [13]. However, the protocol of Doyle and Doyle is more frequently used to extract plant DNA [14,15]. The method developed by Doyle and Doyle is modify the use of CTAB which is used by previous researcher, with advantages of using fewer samples [17,18]. CTAB (exadecyltrimethylammonum bromide) is a cationic detergent and has properties of destroying cells, breaking down proteins, and separating carbohydrates from nucleic acids[. When the tissue destruction process occurs, the polyphenol compounds from the vacuole are oxidized and then covalently bind with proteins and nucleic acids so that the extracted DNA solution will turn brown [16-18]. Since several Citrus have their own characters relating with their metabolic compound, the aim of this study was to apply the Doyle and Doyle to compare their quality and quantity. The research will find out whether this method is good for obtaining good plant DNA from several species of Citrus.

2. Materials and method

2.1 Materials

The materials used in the study is Pamelo Yellow Orange (*Citrus maxima* Merr), Lime Orange (*C. limon*) and Sunkist Orange (*C. sinensis*) from West Java. The DNA isolation will use 400 µg of leaf from each Citrus.

2.2 DNA isolation

DNA isolation was performed according to Doyle and Doyle method [17,18] with slight modification. DNA isolation buffer using the Doyle & Doyle method consist of buffer CTAB 2%, 70% ethanol, CIA (Chloroform–Isoamyl–Alcohol), and TE buffer. The modification was conducting by adding polyvinylpyrrolidone (PVP) 0.01 g; Isopropanol; and Sodium acetate (Na–Acetate) 3M. Further modification was eliminate merchaptoethanol, ammonium acetate, EDTA, RNase and Sodium chloride from the DNA isolation process. Ethanol and isopropanol were used to precipitate DNA by recovering DNA from aqueus solution in the presence of monocovalent like sodium. The elimination of EDTA was conducted since it may coprecipitate with the DNA of Citrus.

2.3 Analiysis of quality and quantity of DNA

A quantitative test using NanoDrop is carried out to measure the concentration and purity of DNA Citrus. The quality and quantity of DNA were analysed using wavelength 260 nm and 280 nm.

3. Result and discussion

The establishment of suitable nucleic acid isolation procedures for Citrus that can produce high quality and quantity of genetic material with minimal contaminant from every part of the plant is a crucial starting point for subsequent molecular genetic studies. DNA isolation on *Citrus* leaves using the Doyle and Doyle method with the addition of CTAB which is used to dissolve the plasma membrane and form bond complexes with fructants and other polysaccharide compounds which can later be removed during the addition of chloroform compounds. DNA samples from yellow grapefruit, lime, and sunkist leaves that have been isolated have resulted in white color samples. The color indicates that there is no polyphenol oxidation in DNA of Citrus at the tube [14,15]. Concentration of polyphenol in Yellow Bali oranges leaves were 0,61-22,24 mg GAE/g[19,20], lime 3,83 mg GAE/g[21,22] and sunkist 3.22 mg GAE/g[23]. The concentration values of the three samples were above 100 ng/µl. Interestingly, based on the results of measurements of DNA concentration in Table 1., it was found that the higher the DNA concentration, the smaller the polyphenol concentration in Citrus leaves. The process of eliminating polysaccharide compounds is an important step because polysaccharides often settle together with DNA, causing the texture of the DNA solution to become thick and sticky [15]. In DNA isolation of *Citrus* leaves, a buffer solution and alcohol group were also used to remove contaminants in DNA sample. The use of buffer solutions in the isolation process uses Tris HCl with pH is 8. The function of the buffer solution is to maintain the DNA structure during the lysis and purification process. In the final stage, giving TE buffer serves to keep DNA intact during storage [15–18].

Table 1. DNA Spectrophotometer results for Yellow Bali Oranges, Lime, and Sunkist leaves

Sample	Consentration (ng/µl)	A_{260}	A_{280}	Purity (A_{260}/A_{280})
Citrus maxima Merr.	429.7	8.594	4.452	1.93
Citrus limon	664.5	13.289	7.382	1.8
Citrus sinensis	888.6	17.772	11.796	1.51

DNA is classified as good quality if based on the nanodrop test it has a purity of 1.8 - 2.0. Therefore, DNA isolation of Sunkist oranges with a purity of 1.51 means that it is still contaminated by protein, while DNA isolation of yellow grapefruit and lime is not contaminated by RNA and protein. The number of purity that have a ratio value of A_{260} / A_{280} less than 1.8 indicate the presence of phenol contaminants, protein, salts, proteins or polysaccharides compounds that are carried along during the extraction process [23]. Other study supported the result with Yellow Bali Oranges and Lime which showed that isolation of DNA from Citrus leaves always gaining purity of DNA value of 1,7–1,9 [16,24]. Since the concentration of polyphenol of sunkist was highest about 3.22 mg GAE/g[25] comparing with other samples, it showed postive correlation with DNA purity.

The classic Doyle and Doyle method procedure uses liquid nitrogen for the isolation of plant DNA. However, the use of liquid nitrogen is not environmentally friendly as well as the price is relatively expensive, so the Doyle and Doyle method is modified by removing or replacing liquid nitrogen with other materials that are cheaper and environmentally friendly. Plant DNA isolation procedures generally suggest the use of liquid nitrogen during crushing plant tissue samples[26]. Liquid nitrogen functions to freeze the tissue making it easier to scrape. Liquid nitrogen is rarely used in developing countries because it is expensive and requires refrigeration and special tanks for storage. Based on the

research, the use of liquid nitrogen did not significantly affect the results of DNA isolation, so the use of liquid nitrogen was not absolutely necessary.

Previous research which modified the CTAB method by removing liquid nitrogen and increasing the concentration of PVP, sodium metasulfite, sucrose, and ascorbic acid in Citrus plant isolation range of DNA purity was gaining purity 1.9 and DNA concentrations range from 100-500 ng/µl, the CTAB modification method by comparing the classic CTAB method against several agricultural plants such as *Zea mays, Oryza sativa, Solanum tuberosum, Capsium annuum , Cucurbitales maxima, Cucumis sativus, Lupinus lupinus, Lens culinaris, Triticum aestivum,* and *Gossypium arboretum* showed that the classical CTAB method had an average DNA purity below 1.8, while the modified method DNA purity values were 2.08-2.33[15–17]. However, the two methods used were similar in the value of the concentration of DNA samples in agricultural crops [27].

Isolation of orange DNA in this study using the modified Doyle and Doyle method can produce good DNA purity and concentration. Among a lot of methods which are used to extract DNA for Citrus, this protocol of DNA extraction is cost effective and efficient. Removal of polysaccharides and polyphenol from citrus plants leaves is easy by using this method. The modification of the Doyle and Doyle method was also potentially applicable for other plant types that have high polysaccharides content. Hopefully, these methods will also applicable for many other aromatic and herbal plants that rich in polyphenol and polysaccharides.

4. Conclusion

In this research, the assessment of DNA isolation on the leaves of yellow bali grapefruit, lime, and sunkist using Doyle and Doyle method gaining high quantity and normal quality of DNA. The quality and quantity of DNA also showed correlation with the amount of polyphenol in leaves. The quality of DNA were of proved that polyphenol in leaves can inhibit the DNA isolation in leaves. However, the DNA isolation using application of Doyle and Doyle methods with sligh modification was suitable to apply in yellow bali grapefruit, lime, and Sunkist and potentially applicable to other species.

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