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Comparative Efficacy of Two Extraction Methods for Determining Chlorophyll Pigments in Thirty Underutilized Horticultural Species

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Abstract

A number of methods involving different solvents have been employed for determination of chlorophyll content in various species. In the present study, the efficacy of two most commonly used solvents viz. acetone and dimethyl sulphoxide in extraction of total chlorophylls was compared in thirty perennial, tropical species of horticultural importance. Results revealed that no single solvent was suitable for all the species and its suitability varied greatly among the species, even those belonging to same family or genus. Hence, for each new species in question, suitable method should be identified. Results obtained in the present study could be used as ready reckoners for the described 30 species for conducting further researches involving photosynthetic pigment determination.

Key words: *Acetone, chlorophyll, Crop Wild Relatives, DMSO, underutilized fruits*

Introduction

Perennial horticultural species have long been known to have economic and ecological significance. Apart from the commercial crops, a number of lesser known and regionally popular horticultural species are being utilized in various parts of the world. Of these, underutilized fruit species provide livelihood and nutritional security to native population apart from offering their ecosystem services (Singh et al., 2012). On the other hand, crop wild relatives (CWRs) have gained increasing importance in the recent past for improving the productivity of commercial horticultural crops (Waman et al., 2018). These species mostly have restricted geographical distribution and scientific information about them is generally scanty. Nevertheless, researchers across the globe have started paying attention to these untapped reservoirs of novel genes (Ford-Lloyd et al., 2011).

Studies have indicated that underutilized horticultural genetic resources could be potential new crops, gene sources, sources of medicines, natural dyes etc. (Bohra et al., 2018). However, in order to harness their potential, systematic studies on various aspects of physiology, crop production, crop improvement and crop protection need to be undertaken. Chlorophylls are known to play an important role in the primary metabolism of plants and are considered as an essential component in most of the studies dealing with these aspects (Mahajan and Pal, 2016; Minocha et al., 2009). Acetone, methanol, ethanol, dimethylsulfoxide (DMSO), N, N- dimethyl formamide (DMF), chloroform etc. have been commonly used solvents for extraction of chlorophyll pigments in different species. Although, several methods and solvents have been reported for its determination; each of these have their pros and cons. For example, use of organic solvents such as acetone generally involves procedures having multiple steps and results in loss of pigments during the process; whereas, solvents like DMSO are considered harmful, if not handled with care (Minocha et al., 2009; Stiegler et al., 2005).

Though a number of reports describing effect of solvents on extraction efficacies are available, meagre work has been done in perennial hardwood species (Minocha et al., 2009). Considering this, present study was undertaken to identify the most suitable method for estimation of total chlorophylls in 30 tropical species including underutilized fruits, commercial crops and their wild relatives. Most of these species are distributed in different tropical parts of the world and hence, the identified methods could be used for various studies requiring pigment determination in these species.

Materials and methods

Collection of Samples

Details of selected species and their present uses/ future utilization potential have been presented in Table 1. Leaf samples of the listed species were collected from underutilized fruits germplasm block and experimental fields of ICAR - Central Island Agricultural Research Institute, Port Blair, India. Recently matured leaves, free from disease or pest incidence were selected for the study. Leaves were gently wiped using wet tissue paper before use to remove any adhering dirt. Mid-rib was removed and lamina from minimum three leaves in each species was bulked and used for obtaining desired sample.

Extraction using Method I

One gram of leaf sample was cut into small pieces using a stainless steel scalpel and grinded in dark condition with 80% pre-chilled acetone (Merck, Mumbai, India) till residue became colourless. Volume was made up to known value using 80% acetone and extract was centrifuged at 5,000 rpm in a cooling centrifuge (Eppendorf, Germany) at 4 ºC for 5 min. Supernatant was carefully removed and used for measurement of absorption at wavelengths of 646 and 663 nm using Biospectrometer (Eppendorf, Germany).

Extraction using Method II

Leaf sample was cut into small pieces using a scalpel and 125 mg sample was placed in a test tube containing 6.25 ml of DMSO (HiMedia, Mumbai, India). Sample tubes were capped and protected from light by covering them with aluminium foil before incubation for 72 h at room temperature (28 ± 2 °C). Supernatant obtained from each sample was used for measurement of absorption at wavelengths of 649 and 665 nm using Biospectrometer.

Estimation of Chlorophyll and Data Analysis

Total chlorophyll content was determined using the equations given by Wellburn (1994) as given below and obtained values (µg/ml) were converted to mg/g of leaf tissue. Values were expressed as mean ± standard error. Data obtained was subjected for *t*-test using Web Agri Stat Package (WASP 2.0, ICAR-CCARI, Ela, India).

Method I: $\text{Total Chlorophyll (µg/ml)} = (12.21\ A_{663} - 2.81\ A_{646}) + (20.13\ A_{663} - 5.03\ A_{646})$

Method II: $\text{Total Chlorophyll (µg/ml)} = (12.19\ A_{665} - 3.45\ A_{649}) + (21.99\ A_{649} - 5.32\ A_{665})$

Results and discussion

Thirty tropical horticulturally important species (Table 1) were selected for the present study. General utilization pattern of the species has also been presented which revealed that most of the fruit species are mainly consumed as fresh fruits, while some of them such as *Aegle marmelos, Morinda 'citrifolia' 'etc'*. have also been valued for their medicinal properties. Species such as *'Averrhoa' 'bilimbi'* and *Garcinia cowa* have traditionally been used in the preparation of culinary dishes as acidulant. Uses of CWRs range from medicine, rootstocks to potential gene sources as indicated in Table 1. Commercial crops such as cashew, mango, mangosteen, arecanut, nutmeg and jamun were also selected to compare them with their wild relatives. Popular exotic fruit species being popularized in India such as velvet apple, West Indian cherry, bread fruit, strawberry guava, milk fruit etc. were also studied.

Chlorophyll content has often been used as an index of potential dry matter production (Whittaker and Marks, 1975; Paliwal et al., 1986) as chlorophyll is essential for photosynthesis, a process which is central to a plant's physiological functions. Leaves, being primary site of photosynthesis, are the most suitable plant part for estimating the concentration of photosynthetic pigments in a plant. Leaf age is an important factor for chlorophyll content (Kamble et al., 2015), wherein matured leaves generally have higher chlorophyll concentration than younger ones (Datta et al., 2018). Hence, only mature leaves were used for analysis during present study.

Table 1. List of horticultural species and crop wild relatives used in the present study

Family	Scientific name	Important uses
Anacardiaceae	*Anacardium occidentale* L.	Nuts as commercial dry fruit
	Semecarpus kurzii Engler	Fruits consumed by the *Onge* tribe, used in traditional medicine
	Semecarpus prainii King	Potential for crop improvement of cashew
	Mangifera indica L.	Commercial fruit crop
	Mangifera camptosperma Pierre	Fruits consumed by tribes
Arecaceae	*Areca catechu* L.	Masticatory nut, commercial plantation crop
	Areca triandra Roxb.	Masticatory nut used by tribals
Clusiaceae	*Garcinia mangostana* L.	Commercial fruit crop
	Garcinia cowa Roxb.	Rootstock for mangosteen; rind used as acidulant spice
	Garcinia dhanikhariensis S.K. Srivast.	Edible fruits, source of anthocyanins
	Garcinia xanthochymus Hook. f. ex T. Anderson	Edible fruits, source of gamboge
Dilleniaceae	*Dillenia indica* L.	Chutneys, traditional dishes
Ebenaceae	*Diospyros blancoi* Willd.	Fresh consumption
Euphorbiaceae	*Averrhoa bilimbi* L.	Pickle, acidulant
Flacourtiaceae	*Flacourtia indica* (Burm. f.) Merr.	Fresh consumption
Malpighiaceae	*Malpighia glabra* L.	Fresh consumption
Moraceae	*Artocarpus altilis* (Parkinson) Fosberg	Culinary purpose
Myristicaceae	*Myristica fragrans* Houtt.	Nuts and mace are used as spice
	Horsfieldia glabra (Blume) Warb.	Nuts are source of energy
	Knema andamanica (Warb.) W. J. de Wilde	Rootstock for cultivated nutmeg
	Myristica andamanica Hook f.	Nuts in traditional medicine; source of fat
Myrsinaceae	*Ardisia solanacea* Roxb.	Fresh consumption
Myrtaceae	*Syzygium cuminii* (L.) Skeels	Commercial fruit crop
	Syzygium claviflorum Wall. ex A.M. Cowan & Cowan	Edible fruits
	Syzygium malaccense (L.) Merr. & Perry	Edible fruits
	Syzygium jambos (L.) Alston	Beverages
	Psidium cattleianum Sabine	Fresh consumption
Rutaceae	*Aegle marmelos* (L.) Correa	Fresh consumption, beverages, medicines
Rubiaceae	*Morinda citrifolia* L.	Medicinal formulations
Sapotaceae	*Chrysophyllum cainito* L.	Fresh consumption

Table 2. Comparative efficacy of two extraction methods on recovery of total chlorophylls (mg/g, FW) in selected horticultural species and crop wild relatives

No.	Scientific name	Solvent		*t*-test	Change (times over first method)
		80% Acetone	DMSO		
1.	*Anacardium occidentale*	4.38 ± 0.041	1.86 ± 0.038	**	0.43
2.	*Semecarpus kurzii*	6.81 ± 0.096	9.44 ± 0.106	**	1.39
3.	*Semecarpus prainii*	5.41 ± 0.030	2.77 ± 0.009	**	0.51
4.	*Mangifera indica*	4.46 ± 0.047	8.08 ± 0.044	**	1.81
5.	*Mangifera camptosperma*	2.67 ± 0.055	1.36 ± 0.014	**	0.51
6.	*Areca catechu*	4.05 ± 0.058	1.60 ± 0.004	**	0.40
7.	*Areca triandra*	3.40 ± 0.010	1.89 ± 0.011	**	0.56
8.	*Garcinia mangostana*	3.55 ± 0.014	4.68 ± 0.038	**	1.32
9.	*Garcinia cowa*	5.38 ± 0.037	7.15 ± 0.113	**	1.33
10.	*Garcinia dhanikhariensis*	1.61 ± 0.008	2.43 ± 0.008	**	1.51
11.	*Garcinia xanthochymus*	5.65 ± 0.029	2.89 ± 0.035	**	0.51
12.	*Dillenia indica*	1.28 ± 0.008	0.41 ± 0.004	**	0.32
13.	*Diospyros blancoi*	0.20 ± 0.007	0.39 ± 0.005	**	1.97
14.	*Averrhoa bilimbi*	0.14 ± 0.017	0.44 ± 0.003	**	3.11
15.	*Flacourtia indica*	0.44 ± 0.031	0.54 ± 0.007	*	1.24
16.	*Malpighia glabra*	0.14 ± 0.007	0.48 ± 0.006	**	3.51
17.	*Artocarpus altilis*	1.31 ± 0.005	0.29 ± 0.004	**	0.22
18.	*Myristica fragrans*	2.63 ± 0.011	1.94 ± 0.004	**	0.74
19.	*Horsfieldia glabra*	3.28 ± 0.018	7.56 ± 0.046	**	2.31
20.	*Knema andamanica*	4.80 ± 0.010	6.18 ± 0.057	**	1.29
21.	*Myristica andamanica*	4.74 ± 0.029	3.44 ± 0.027	**	0.73
22.	*Ardisia solanacea*	0.63 ± 0.010	0.30 ± 0.004	**	0.48
23.	*Syzygium cuminii*	8.50 ± 0.018	2.91 ± 0.034	**	0.34
24.	*Syzygium claviflorum*	5.85 ± 0.016	3.24 ± 0.006	**	0.55
25.	*Syzygium malaccense*	2.50 ± 0.013	1.94 ± 0.006	**	0.78
26.	*Syzygium jambos*	0.69 ± 0.019	0.26 ± 0.006	**	0.38
27.	*Psidium cattleianum*	0.13 ± 0.009	0.33 ± 0.003	**	2.58
28.	*Aegle marmelos*	0.16 ±0.017	0.46 ± 0.008	**	2.82
29.	*Morinda citrifolia*	0.92 ± 0.027	0.27 ± 0.010	**	0.29
30.	*Chrysophyllum cainito*	0.17 ± 0.002	0.31 ± 0.007	**	1.89

* and **: Significant at 5% and 1% level of significance, respectively

It is known that recovery of photosynthetic pigments is considerably affected by the extraction process and hence, two most commonly used solvents were compared for their efficacies. In order to identify the superior method for extracting chlorophyll pigment in each species, paired *t*-test was performed, which revealed species-specific results and interestingly, even species belonging to the same family had different solvent suitability. In general, for sixteen species, traditional maceration based method involving acetone (80%) as solvent was suitable, whereas DMSO was ideal for remaining fourteen species. Such variations in the efficacy of pigment extraction have been attributed to the difference in solubility of pigments or its affinity towards different chemical solvents (Vimala and Poonghuzhali, 2013).

In some cases, species belonging to the same genus showed different solvent suitability e.g. among the species of Anacardiaceae, acetone maceration was observed to be better method for a species of wild cashew (*Semecarpus prainii*), while the solvent was not suitable for another wild cashew (*Semecarpus kurzii*). Similarly, solvent suitability was different for mango (*Mangifera indica*) and wild mango (*Mangifera camptosperma*). On the contrary, in a number of cases, same solvent was found suitable for different species of a genus e.g. acetone mediated extraction was recommended for members of Arecaceae (*Areca catechu* and *A. triandra*), Myristicaceae (*Myristica fragrans* and *Myristica andamanica*) and Myrtaceae (*Syzygium cuminii*, *Syzygium claviflorum*, *Syzygium malaccense* and *Syzygium jambos*). Similarly, DMSO was observed to be a better solvent for extraction of total chlorophylls from members of Clusiaceae (*Garcinia mangostana*, *Garcinia cowa*, *Garcinia dhanikhariensis*), with an exception of *Garcinia xanthochymus,* in which acetone method was superior. In remaining six species, DMSO was found to be promising solvent, while in five, acetone extraction was superior. Earlier report on hardwood and conifer species suggested that acetone extraction was suitable for seven species, while DMSO was found suitable in other cases (Minocha et al., 2009). Present results are also in accordance with the earlier reports which suggested that choice of method/ solvent for chlorophyll extraction varies with the species in question (Manuela et al., 2012) and sometimes, even among the varieties within a single species (Krishnan et al., 1996).

The efficacy of each solvent varied considerably as total chlorophyll recovery in DMSO method ranged between 0.22 times (*Artocarpus altilis*) to 3.51 times (*Malpighia glabra*) over acetone method. Great variations in extraction efficacies have been reported by earlier researchers in which DMSO was rated 0.79 to 2.44 times (Nikolopoulos et al., 2008) and 0.81 to 2.20 times (Minocha et al., 2009) efficient over acetone. Low efficacy of DMSO in most of these species with thick/ glossy leaves is in line with earlier reports which suggested that incubation alone would not be sufficient for extracting total chlorophylls from tissues and maceration is required (Tait and Hik, 2003). Considering less than half recovery of total chlorophyll content in case of *Anacardium occidentale*, *Areca catechu*, *Dillenia indica*, *Artocarpus altilis*, *Ardisia solanacea*, *Syzygium cuminii*, *Syzygium jambos* and *Morinda citrifolia*, use of DMSO as solvent should be discouraged. Similarly, acetone extraction should be avoided in *Averrhoa bilimbi*, *Malpighia glabra, Horsfieldia glabra*, *Psidium cattleianum* and *Aegle marmelos*.

Each solvent has some limitations as cited in many instances. In case of acetone, some quantity of solvent is lost during extraction and estimation due to its high vapour pressure, thereby providing datasets with variable results at times (Stiegler et al., 2005). Similarly, extraction using DMSO does not involve any manual maceration and hence, extraction efficacy largely depends on its diffusivity inside the tissues that would not be same in species with homobaric and heterobaric leaves (Nikolopoulos et al., 2008).

Determination of photosynthetic pigments has wide scale applicability as it has been commonly employed in studies pertaining to various aspects of agri-horticultural crops. Chlorophyll content is commonly determined to study the effect of growing conditions on nutritional and physiological status of plants (Mahajan and Pal, 2016); to evaluate plant health (Minocha et al., 2009); to identify germplasm for drought (Ramalaxmi 2011) and salinity (Upadhyay 2016) tolerance, to facilitate domestication of new species (Bohra et al., 2018) and many other related aspects.

Conclusion

It could be concluded that suitability of solvent for extraction of photosynthetic pigments varied greatly among the species, genus and family. The results obtained in the present study could serve as ready reckoner for the discussed thirty species.

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Disclosure statement

Authors declare that they have no conflict of interest.

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