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DNA extraction protocols for *Citrullus lanatus* and *Capsicum frutescens*: Effects of incubation temperatures and ethanol concentrations on DNA purity and quantity

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Citrullus lanatus **has exceptional levels of oil (42 – 57%) and protein (33.8%) while** ***Capsicum frutescens*** **has high quantities of pro-vit A, C, B (B_1, B_2, B_3). In spite of these endowments, no disease resistant variety of** ***C. lanatus*** **has been developed in Nigeria through conventional breeding. Likewise,** ***C. frutescens*** **improvements has been limited mainly by susceptibility to pepper veinal mottle virus which is incurable and which leads to huge economic losses. Molecular breeding is a tested technique for developing disease-resistant varieties of species. A fundamental step to any molecular biology study is the capability to isolate pure genomic DNA. Many of the published plant DNA extraction protocols are not suitable for all plants due to the presence of secondary metabolites. Since every step of a protocol should be optimized for each species, this study which is the first in a series was, therefore, undertaken to ascertain the best incubation temperature and ethanol concentration that can be suitable for DNA extraction from both species. Results showed that the following combinations were optimal for** ***C. lanatus*** **: 100%ethanol/60°C and 95%/60°C. Their A_{260}/A_{280} ratios were 1.950 ± 0.014 and 1.860±0.031, respectively, while their corresponding DNA concentrations were 3.087 and 2.973 µg/µl. For** ***C. frutescens,*** **the combinations, 100%/60°C and 95%/60°C were optimal and their A_{260}/A_{280} ratios were 1.963 ± 0.004 and 1.803 ± 0.053 respectively while their corresponding DNA concentrations were 2.973 and 2.820 µg/µl. So it is recommended that any of the combinations for each species could be used for DNA extraction.**

Key words: DNA, extraction, protocol, pepper, egusi.

INTRODUCTION

Citrullus lanatus commonly known as egusi melon belongs to the family Cucurbitaceae. The largest producers are countries in West and Central Africa particularly Nigeria (Van der Vossen et al., 2004). The egusi seed is both protein and oil-rich. Reports have indicated that it contains about 42 – 57% oil (Fokou et al., 2004) and 33.8% protein (Ogbonna, 2013).

Egusi seed has mean total nitrogen of 5.75%. This is higher than those of peanut and cowpea but slightly less than that of soybean (6.65%) (National Academy of Sciences, 2006). The advantage egusi seed, however has over soybean is that it has no antinutrients (NAS, 2006) which make it particularly convenient to be used as a livestock feed (Van der Vossen et al., 2004). The other nutritional compositions of egusi are outstanding. It has exceptional levels of the essential amino acids, arginine,

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methionine and tryptophan. It has good quantities of the vitamins, B1, B2 and niacin as well as the following minerals, phosphorus, potassium, magnesium, manganese, sulphur, calcium, iron and zinc (NAS, 2006).

Due to the potentials of this plant, NAS (2006) reported that it deserves concentrated local, regional and international attention. The authors further listed several areas of research needed on the plant among them is the issue of taxonomic clarification to determine if egusi is an aberrant watermelon. On this issue, NAS (2006) suggested the use of DNA fingerprinting technique and carefully monitored cross-pollination trials to clarify whether egusi is an inedible watermelon or a distinct species. Furthermore, van der Vossen et al. (2004) reported that breeding programmes in egusi will be greatly augumented through the use of molecular marker assisted selection and genetic transformation. These techniques may lead to the development of resistance to diseases and pests which has so far remained unattainable through conventional breeding.

Capsicum frutescens widely known as pepper belongs to the family Solanaceae. Generally it is regarded as the world's second most important crop after tomato (Yoon et al., 1989). It has been noted to be a very popular spice that is used all over the world (Mohammed et al., 2013). The largest producer in Africa is Nigeria which accounts for 50% of total Africa production (Abu et al., 2013). The many uses of pepper have been listed by various authors. These include: The use of pepper as a flavouring and colouring agents in food manufacturing and cosmetics industries (Bosland and Votara, 2000); as a medicine to combat constipation, relieve pain among others (Dagnoko et al., 2013); as a very important component in the preparation of soups, stew and other foods in many Nigerian homes (Onwubuya et al. 2008).

Nutritionally, pepper is highly valued. It contains high quantities of pro-vitamin A, C, B (B1, B2, B3), k, calcium, iron,zinc and fibre. Two species are widely grown in Nigeria and these include *C. frutescens* which is pungent. It has two varieties, bird pepper commonly called 'atawere' in Nigeria and Cayenne pepper or red pepper popularly known as 'Sombo'. The other species is *Capsicum annum* which is less pungent and mild. It also has two varieties, 'atarado' and 'tatase'. Other variations among these are in terms of colour, size and shape (Madu and Uguru, 2006; ICS-Nigeria et al. 2013).

Inspite of the acres of land reserved for pepper production in Nigeria, low yields have been reported by many authors (Adigun, 2000; Jaliya and Sani, 2006). Its production constraints have been limited mainly to different types of pathogenic organisms and insect pests (Madu and Uguru, 2006; Idowu-Agida et al., 2010; Mohammed et al., 2013). Among the disease organisms, the most virulent and of very serious concern to farmers is the pepper veinal mottle virus (PVMV) which is incurable (Madu and Uguru, 2006). The authors further observed that the viral infections can lead to huge economic losses and suggested that the development of resistant varieties is the most likely cost effective means of control of PVMV.

All these point to the fact that the use of molecular breeding techniques is the best option for the improvement of *C. lanatus* and *C. frutescens.* A fundamental step to any molecular biology study is the capability to isolate genomic DNA (Sharma and Purohit, 2012). The authors further noted that the isolated DNA must be of sufficient molecular weight and purity to be suitable for polymerase chain reaction (PCR) and restriction analysis. Successful extraction of such DNA could lead to the development of DNA fingerprinting techniques which could be used as a diagnostic tool for the assessment of genetic variability of a particular species (Vinod, 2004) and in this case for *C. lanatus* and *C. frutescens.*

Porebski et al. (1997) observed that the published DNA extraction protocols are not reproducible for all species judging from the number of species specific protocols being reported. Sangwan et al. (1998) went further to state that the DNA isolation methods need to be adjusted even to each plant tissue due to the presence of secondary metabolites. Vinod (2004) suggested that researchers working with minor crops or unexploited crop species which have no established DNA extraction protocol, should try the various published protocols, do some adjustments in order to derive a suitable protocol for the particular species. This research was, therefore, undertaken to study the effects of three temperature regimes and three ethanol concentrations on the purity and quantity of DNA extracts from *C. lanatus* and *C. frutescens.*

MATERIALS AND METHODS

The seeds of the two species were purchased from Nsukka market Enugu state Nigeria. They were authenticated by Mr Alfred Ozioko of Biodiversity and Conservation Programme/International Centre for Ethnomedicine and Drug Development located at No. 110 Aku Road, Nsukka. The experimental design is as shown in Table 1. The extraction procedure was adapted from the Department of Microbiology, University of Nigeria, Nsukka, Molecular Biology Workshop Manual of 2011 (Ezeonu, 2011). The same steps were used for all the treatment combinations in Table 1.

The seeds of the respective species were separately ground into powder using mortar and pestle. Subsequently, 1 g of the respective powder was transferred into each of 27 Eppendorf tubes into which were added 2 ml of sodium dodecyl sulfate (SDS) homogenization buffer (10mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 0.5% SDS). The mixture of powder and SDS was incubated in a water bath for 15 min at the following temperature regimes: 10, 30 and 60°C. Nine tubes from each of the species were respectively incubated at each temperature regime. At the end of the incubation time, the respective tubes were cooled on ice in an ice bath for another 15 min. Subsequently, the homogenate were centrifuged at 12,000 rpm for 5 min. The respective supernatant were collected and transferred into new Eppendorf tubes. Subsequently, 40 µl of chloroform was added to the supernatant by allowing it to run down the side of the respective tubes. The tubes were swirled gently taking care not to mix the two liquids. The denatured proteins formed a white layer on top of the organic chloroform layer and the swirling of the tubes continued for about 2 to 3 min. With the aid of a dropper pipette, the top aqueous layers (homogenate) were transferred carefully into new Eppendorf tubes and the chloroform

Table 1. Experimental Design (Three replications for each treatment combination).

Ethanol concentration (%)	*C. lanatus*			*C. frutescens*		
	Temperature (°C)					
	10	30	60	10	30	60
70	1	1	1	1	1	1
	2	2	2	2	2	2
	3	3	3	3	3	3
95	1	1	1	1	1	1
	2	2	2	2	2	2
	3	3	3	3	3	3
100	1	1	1	1	1	1
	2	2	2	2	2	2
	3	3	3	3	3	3

Figure 1. DNA precipitate.

was poured into a clearly labeled waste container. This procedure was repeated four times for a total of four chloroform extractions. After the last extraction, the aqueous phase was collected leaving a small amount behind to avoid transferring any chloroform to the new tube.

The Eppendorf tubes containing the aqueous layers were allowed to cool on ice for 10 min. Subsequently, 1 ml of respective cold ethanol (70, 95 and 100%) was poured slowly down the side of the tubes. Each concentration of ethanol was added to three tubes from each of the temperature regimes for each of the species. A few minutes later, white stringy precipitate began to form on top of the ethanol in each tube (Figure 1). The DNA precipitate was centrifuged at 12,000 rpm for 8 min. The supernatant was decanted and the pellet was allowed to air-dry. Fifty microliter (50 µl) of TE buffer was added to the DNA precipitate and the tube was stored in a refrigerator.

Subsequently, 5 µl of each DNA extract was diluted in 1 ml of deionized water and was mixed gently. The dilution was transferred to a spectrophotometer cuvette. The spectrophotometer was zeroed at 260 nm with 1 ml of deionized water in an empty quartz cuvette. The cuvettes containing the diluted DNA extracts were then inserted into the spectrophotometer and the absorbance was read at 260 nm. The same procedure was repeated for 280 nm. The assessment of the purity of each of the DNA extracts was done by calculating the ratio of A_{260}:A_{280} (Held, 2001). Pure good quality DNA has A_{260}:A_{280} ratio ≥ 1.8 (Adeel 2008, Oxford Gene Technology, 2011). DNA quantity was obtained using the formular below:

DNA conc (µg/ml) = A_{260} reading x dilution factor x standard value.

The quantities of DNA obtained in this study were compared with the range (2.5 – 5.0 µg) reported by Lipp et al. (2005) for spectrophotometric analyses.

RESULTS

It could be observed from Table 2 that there were highly significant differences in the effects of the ethanol regimes, temperature concentrations and their combinations on the purity of DNA extracted from *C. lanatus* and *C. frutescens.* Table 3 shows that for each of the species, as the temperature and ethanol concentration increased, the DNA purity increased. The F-LSD calculated at 0.05% level of probability, however, revealed that some purity levels were not significantly different from one another while some were. For both species, pure DNA were produced by the following combinations: 100% ethanol / 60°C and 95%/60°C. Thus, 60°C incubation tempera-ture was the best. The purity levels for these various combinations were significantly different from one another and the purest DNA was obtained at 100% / 60°C. The most impure DNA were obtained at 10°C at all the ethanol concentrations and 30°C for 95 and 70% ethanol.

The ANOVA in Table 4 reveals that for each of the species, the quantities of DNA obtained were highly significantly different for the three ethanol concentrations and the three temperature regimes. Table 5 shows that for both species, as the temperature and ethanol concentration increased, the quantity of the DNA extract increased with the exception of quantity obtained at 95%/10°C for *C. frutescens.* The F-LSD calculated at 0.05% level of probability again showed that some DNA quantities were not significantly different from one another while some were. For both species, the quantities produced at 60°C

Table 2. Analysis of variance of the effects of ethanol concentrations and temperature regimes on DNA purity.

Source of Variation	DF	*C. lanatus*		*C. frutescens*	
		MS	VR	MS	VR
Ethanol	2	0.043	64.082**	0.107	47.852**
Temperature	2	0.589	868.443**	0.507	227.832**
Ethanol x Temp	4	0.008	11.992**	0.17	7.594**
Error	18				

**, Significantly different at 1% level of probability.

Table 3. Mean DNA purity levels for the various ethanol/temperature combinations.

Ethanol concentration (%)	*C. lanatus*			*C. frutescens*		
	Temperature (°C)					
	10	30	60	10	30	60
70	1.303± 0.011^{f}	1.497± 0.027^{e}	1.703±0.015^{c}	1.317± 0.018^{g}	1.363± 0.004^{f}	1.630±0.049^{c}
95	1.330±0.019^{f}	1.583±0.008^{d}	1.860±0.031^{b}	1.317±0.016^{g}	1.463±0.033^{d}	1.803±0.053^{b}
100	1.347±0.004^{f}	1.617±0.020^{d}	1.950±0.014^{a}	1.367±0.054^{e}	1.630±0.012^{c}	1.963±0.004^{a}
F-LSD0.05	0.0449					

For each species, means followed by the same lower case letters are not significantly different at 5% level of probability.

Table 4. Analysis of variance of the effects of ethanol concentrations and temperature regimes on DNA quantity.

Source of Variation	DF	*C. lanatus*		*C. frutescens*	
		MS	VR	MS	VR
Ethanol	2	0.077	16.290**	0.113	51.757**
Temperature	2	1.943	408.515**	1.465	672.757**
Ethanol x temp	4	0.012	2.428**	.022	9.900**
Error	18	0.005		0.002	

** = significantly different at 1% level of probability.

Table 5. Mean DNA Quantities obtained from the ethanol/temperature combinations.

Ethanol (%)	*C. lanatus*			*C. frutescens*		
	Temperature (°C)					
	10	**30**	**60**	**10**	**30**	**60**
70	2.030±0.074^{f}	2.550±0.046^{e}	2.813±0.052^{c}	2.003± 0.041^{f}	2.350±0.046^{e}	2.645±0.011^{c}
95	2.037±0.047^{f}	2.660±0.032^{d}	2.973±0.036^{b}	1.997±0.020^{f}	2.477±0.025^{d}	2.820±0.025^{b}
100	2.077±0.062^{f}	2.787±0.046^{c}	3.087±0.025a	2.033±0.045^{f}	2.660±0.014^{c}	2.973±0.043^{a}
F-LSD$_{0.05}$	0.087			0.0366		

For each species, means followed by the same lower case letters are not significantly different at 5% level of probability.

for all the ethanol concentrations were significantly different from one another but the highest quantities were obtained at 100%/60°C. The quantities obtained at 100%/30°C and 70%/60°C were, however, not significantly different from one another so either of the combinations can be used. Moderate DNA quantities were also produced at 95%/30°C and 70%/30°C for only *C. lanatus.* The lowest quantities which were not signifi-cantly different were obtained at 10°C for all the ethanol concentrations.

DISCUSSION

A fundamental step in any molecular biology programme is the capability to extract DNA (Sharma and Purohit, 2012). The extracted DNA must be of high quality so as not to jeopardize the results of subsequent experiments. According to the study of Ginwal and Maurya (2010), 'good quality DNA is essential to achieve good results in experiments just like reagents.' In this study, genomic DNA was successfully isolated from *C. frutescens* and *C. lanatus* using various combinations of ethanol levels and temperature regimes. The purities and quantities of the extracted DNA varied for both species. The purest DNA for the species was obtained at these combinations: 100%/60°C and 95%/60°C. Sufficient quantities of DNA that fell within the range of 2.5 – 5.0 µg stipulated by Lipp et al. (2005) for spectrophotometric analyses were also obtained using the same combinations of ethanol and temperature.

Khan et al. (2007) and Gupta and Preet (2012) noted that incubation temperature is an indispensible criterion for the production of highly pure DNA that is also of good quantity. The earlier authors had to increase their incubation temperature from 65 to 70°C to be able to isolate highly pure and quantifiable DNA. The later authors performed DNA extraction at room temperature, 45, 50 and 55°C. They obtained the highest quantity of DNA at 55°C.

Ezeonu (2011) noted that the temperature of 60°C helps the SDS homogenization buffer to dissolve the cellular proteins. This is in line with the results obtained in this study. It was only 60°C that led to the extraction of DNA of high purity and quantity. The most impure DNA were obtained at 10°C; 30°C for 70% and 95% ethanol. Contaminants of extracted DNA include RNA, proteins, polysaccharides and polyphenol compounds (Pandey et al., 1996; Porebsk et al., 1997). Many authors have reported that A_{260}/A_{280} ratios less than 1.8 indicate protein or residual phenol contamination while ratios approximately 2.0 suggest RNA contamination (Thermo Scientific, 2013; Wang et al., 2013). Fortunately, none of the DNA extracts had a ratio of 2 but many had ratios less than 1.8. Contamination by phenol can affect the ratio calculations as well as contribute to over estimation of DNA concentration (Promega, 2013; Oxford Gene Technology, 2011).

Polyphenols can be removed by using polyrinylpyrrolidone which bind to them or through the use of high concentration of β-mercaptoethanol (Suman et al., 1999). Proteins are generally removed by denaturation and precipitation with chloroform and /or phenol (Vinod, 2004). But to prevent protein contamination *ab initio*, Owyoung (2013) suggested the inclusion of a protease digestion step prior to alcohol precipitation. Fisher (2013) observed that low DNA yield could be due to insufficient lysis, insufficient disruption and DNA being still bound to the membrane. The author proffered solutions as follows: prolonging the incubation time in lysis buffer, thorough homogenization of the sample preferably in liquid nitrogen, eluting the DNA in higher volumes or repeating the elution step up to three times. The elution buffer should also be preheated to 60°C before the elution process.

Gupta and Preet (2012) reported that SDS protocol was the cheapest among the DNA extraction protocols they compared. The other two were commercial kits – Dneasy kit and DNAzol$^{(R)}$. They also reported that the SDS protocol provided purity level comparable to DNeasy ^{R} kit and that its yield was 1.4 times higher than the comer-cial kits. This justifies the use of SDS method in this work. In conclusion, incubation temperature of 60°C and ethanol concentrations, 100 and 95% could be used for DNA extraction from *C. lanatus* and *C. frutescens.*

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