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Full Length Research Paper

Light quality and plant growth regulators influence pigment production in *Alternanthera brasiliana* calli

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Alternanthera brasiliana **is a medicinal plant with several popular use and high production of betalains. The aim of the present research was to establish a protocol for callus induction and betalain biosynthesis in *A. brasiliana* species, as well as to investigate the effect of different qualities of light in increasing betalain in *A. brasiliana* callus cultures. In this way, the callus initiation and the improvement of secondary metabolites were induced using varying phytohormones concentrations and under the influence of red, white and blue lights. To start the experiments, the plants were cultivated for 30 days in Murashige and Skoog (MS) medium to grow and form internodes. These were inoculated in MS medium supplemented with different combinations of plant growth regulators to find the best combination for medium callus induction (MCI). After another 30 days, the explants were transferred to a medium for betacyanin induction (MBI) containing thidiazuron (TDZ) and α-naphthalene acetic acid (NAA) under blue, white, and red lights. The best medium for *A. brasiliana* callus induction with higher production of betalains, was the MS medium supplemented white indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), before inoculation on the MBI. The blue and white lights promoted callus pigmentation (betalains), whereas the red light was not effective at inducing pigmentation in the calli.**

Key words: Amaranthaceae, betacyanin, betalains, pigments, spectra of light, secondary metabolites

INTRODUCTION

Seeking an improvement in health and quality of life in recent years, the use of synthetic dyes have been sidetracked, due to the potential hazards they generate, such as allergies and induction of various diseases, that way, natural dyes have gained market due to their bioactive properties and applications as health promoters when included in diets (Martins et al., 2017). Carotenoids, anthocyanin, and betalains are examples of plants with

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pigment production; physiologically, this production acts as attractants for pollinators and agents to disperse the seeds (Osbourn, 2017). These pigments have great potential and can be used as food dyes because they present not only coloring attributes, but also properties related to antioxidant, antiradical, anti-inflammatory and cytotoxic, radio protective, neuroprotective, antimicrobial, anti-proliferative and hepato-protective properties, enabling his use in food, cosmetic, and pharmaceutical industries (Georgiev et al., 2008; Georgiev et al., 2010; Biswas et al., 2013; Martins et al., 2017).

Betalains are nitrogenated, water-soluble vacuolar pigments, produced by most of caryophyllales plants and never co-occur with anthocyanins. They are synthesized from L-tyrosine and L-phenylalanine (Osbourn, 2017) and are immonium derivatives of betalamic acid for which the conjugation system 1,7-diazaheptamethine is the chromophore. The carboxylic groups in the betalain structure provide the acidic characteristic to the molecule, thus, making it different from the alkaloid group, which has basic character (Herbach et al*.,* 2006). The yellow-to-orange-colored betaxanthins release green auto fluorescence and are produced by condensation of betalamic acid and amino acids (or amines). Similarly, the violet betacyanins are *O*-glycosides (C-5 or C-6) and come from the spontaneous condensation of betalamic acid and *cyclo*-dopa. The acylation of betacyanins generates its main derivative, betanin (betanidin-5-*O*-β-glucoside) (Gandía-Herrero et al., 2010; Reis et al., 2015).

The great interest in the market of renewable natural products has drawn attention to the technical and commercial viability of a variety of systems, exploring *in vitro* and cell and tissue cultured plants as potential bio-factories of phytochemical products, where there is a potential in making the production more reliable, simple and predictable. The ability of plant cells, calli, and tissues cultivated *in vitro* to produce and accumulate chemicals is important by maintaining carefully a controlled and aseptic environment; these types of cultures can also provide an excellent source for in-depth research of metabolic and biochemical pathway and the possibility to predict how amount can be produced in the cultures during a period of time, something impossible to make in nature (Rao et al., 2002; Namdeo, 2007; Karuppusamy, 2009).

Another important point is the use of elicitors (that is, chemical and stressor agents) during cultivation which can change or induce some metabolic pathways that affect, both qualitatively and quantitatively, the plant secondary metabolism (Karuppusamy, 2009; Othman et al., 2016).

Physical stimulation using light is one of the elicitor agents widely used in plant tissue culture; due to the phytochromes and cryptochromes, plants can perceive a small fraction of light radiation, changing the gene expression and physiological responses and modifying and even maximizing plant development and levels of secondary metabolites as a form of protection (Katerova et al., 2017). Red and blue lights have been used to elicit several phenotypic expressions via perception and signal transduction pathways. In the induction of betalain biosynthesis, the use of light was reported in sugar beet (*Beta vulgaris*), seedlings of *Amaranthus caudatus*, callus of *Portulaca*, cell cultures of *Chenopodium album*, and callus of *Alternanthera brasiliana* (L.) Kuntze and Adachi 1995; Macedo et al., 1999; Silva et al., 2005; Zhao et al., 2010).

A. brasiliana (L.) Kuntz (Amaranthaceae) is a plant broadly distributed in South America, with ability to accumulate pigments as betalains (betacyanins and betaxanthins) and flavonoids. This species is used in traditional medicine for the treatment of several human pathologies as cough, diarrhea, infections, and it has also analgesic activity. Moreover, its activity as an antiproliferative, anti-inflammatory, antiedematous and antioxidant, has been reported. It also shows activity against the herpes simplex virus and, recently, anticonvulsant effect (Lagrota et al.,1994; Macedo et al., 1999, 2011; Facundo et al., 2012; Andreazza et al., 2013; Schallenberger et al., 2017).

Based on the foregoing, *A. brasiliana* offers a promising object for plant biotechnology studies. Thus, the aim of the present research was to establish a protocol for callus induction and betalain biosynthesis in *A. brasiliana* species, as well as to investigate the effect of different qualities of light in increasing betalain in *A. brasiliana* callus cultures.

MATERIALS AND METHODS

Callus induction

The experimental *A. brasiliana* plants were grown for 30 days in a growth chamber under a photon flux density of 22 μmol m^{-2} s^{-1} for a 16-h photoperiod at 25 ± 2°C. Internode segments of 0.1 to 0.2 cm in length were placed in a medium for callus induction (MCI) (Zhao et al., 2010), which consisted of MS basal medium with sucrose 30 g L^{-1}, phytagel 2 g L^{-1}, myo-inositol 100 mg L^{-1}, adenine 0.5 mg L^{-1}, ascorbic acid 3 mg L^{-1}, and different plant growth regulators. Three different combinations of phytoregulators were added: 1 mg L^{-1} kinetin (KIN) and 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; MCI-1); 0.75 mg L^{-1} indole-3-acetic acid (IAA) and 1 mg L^{-1} 2,4-D (MCI-2); and 1 mg L^{-1} 6-benzylaminopurine (BA) and 0.5 mg L^{-1} 2,4-D (MCI-3). Assembled six plates, each containing ten explants, were inoculated and placed in a growth chamber at 25 ± 2°C for 22 days in the dark, passed this time, and were transferred to white light for an additional 7 days.

Betacyanin induction and light sources

All explants from the MCI media described earlier were transferred to a medium for betacyanin induction (MBI) (Zhao et al., 2010), composed of MS medium supplemented with 3 g L^{-1} phytagel, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 0.5 mg L^{-1} adenine, 3 mg L^{-1} ascorbic acid, thidiazuron (TDZ) 0.5 mg L^{-1}, and 1 mg L^{-1} of 1-naphthaleneacetic acid (NAA). Over a 40-day period, the plates

containing the explants were placed under different light regimes: white light (full-spectrum-fluorescent tube Sylvania®; 40 W), blue light (peak emission at 470 nm-14 W, compact fluorescent lamp Taschibra®), and red light (peak emission at 660 nm-15 W, compact fluorescent lamp G-light®). The photon flow density for the white, blue, and red-light levels were 25, 12, and 22 μmol m^{-2} s^{-1}, respectively, as measured using a light meter (Hansatech® Quantum Sensor QSRED).

Evaluation of callus pigment appearance and callus pigment intensity

This evaluation was developed seeking a visual quantification of the appearance and intensity of the magenta pigmentation produced in the callus. Numerical scores were given to pigment appearance and intensity in the following manner: 0 (zero), for callus without pigmentation (absence of magenta color); (1) for random magenta dots in callus/low pigment intensity; (2) for approximately 50% of magenta in callus/moderate color intensity, and (3) for nearly 100% magenta in callus/high intensity of coloration visually detected. The scores of 0 to 3 were assigned by four different observers realized in the end of the experiment, after almost 70 days in culture, and the averages of the analysis were used.

Relative callus growth rate (RCGR) and total calli weight (Wc)

RCGR was calculated using the fresh callus weight (FC), as described by Silva and Dobránszki (2015), according to the following equation:

$$RCGR = [(\ln FC_f - \ln FC_i)/(t_f - t_i) \times 10^3],$$

Where, FC_f=final fresh callus weight, FC_i=initial fresh callus weight, t_i=first day of treatment, and t_f=last day of treatment.

The total calli weight (Wc) was calculated at the end of the experiment based on the fresh callus weight (FC) from a Petri dish containing 10 calli.

Betaxanthin quantification

Homogenates using 0.25 g of fresh calli, produced by induction, were obtained by maceration in a porcelain mortar using phosphate buffer at 10 mM (pH 6.0) and 10 mM sodium ascorbate. The homogenates were filtered through cheesecloth and centrifuged at 10000 *g*, for 20 min at 4°C (Gandía-Herrero et al., 2005). The spectrophotometric analyses were performed on a T80 UV/VIS Spectrometer (PG Instruments) kept at 25°C (PTC-2 Peltier Temperature Controller). The betaxanthin concentration calculated using the molar extinction coefficient of miraxanthin (ε=48000 $M^{-1}cm^{-1}$) after measurements was taken at 480 nm (Schliemann et al., 1999).

Total betacyanins quantification

The total amount of betacyanins corresponds to the sum of betanidin and betanin amounts, being the first aglycone and the second glycosylated. To extract betanidin, an acetate buffer at 10 mM and methanol (70:30; v/ v) at a pH 5.0, plus sodium ascorbate of 10 mM were used. To obtain betanin, a phosphate buffer of 10 mM at pH 6.0, plus sodium ascorbate (10 mM) and organic solvents was used. For both extractions, 0.25 g of fresh calli was weighed, and the homogenates were produced as described for the betaxanthin quantification. The molar extinction coefficients ε=54000 $M^{-1}cm^{-1}$ and ε=65000 $M^{-1}cm^{-1}$ were employed to calculate the amount of betanidin and betanin, respectively. A wavelength of 536 nm was used as described previously (Gandía-Herrero et al., 2005).

Total flavonoids quantification

The quantification of total flavonoids was done as described by Salvador et al. (2006), using an acetate/methanol buffer as the extraction solvent and the measurements were done at wavelength of 330 nm. The results were expressed as the amount of quercetin (μmol) per g of calli, using the linear equation: $y = 0.1548x - 0.005$ ($r^2 = 0.9952$).

Normalized index of variation (NIV)

NIV was calculated using the equation (Tattini et al., 2006):

$$NIV = (X_{Light} - X_{Control})(X_{Light} + X_{Control}),$$

Where, X_{light} is the two tested light colors (blue and red) and $X_{Control}$ is the white light. The calculations were performed separately for each type of medium used. The NIV depicts the actual treatment effect of the analyzed calli and it may be positive or negative as compared to the control (white light).

Statistical analysis

The experiment was completely randomized in a factorial 3 × 3 design, represented by the three different media cultures and the three light levels. A total of six replications (6 Petri dishes) were performed, with experimental unit being a Petri dish with ten explants. The assays were performed three times and the statistical analyses, ANOVA and Tukey's tests were performed at $P < 0.05$ to calculate significant differences in treatments, using the statistical software SAS v.9.3 (SAS Institute Inc., Cary, NC) (SAS, 2003).

RESULTS

Evaluation of callus pigment appearance and callus pigment intensity

The first changes in coloration of the calli were observed at the beginning of the experiment. Coloration intensified when the samples were placed under the different lights. The calli cultivated in the darkness were white in color and after exposure to different lights turned green (Figure 1G and H), pink (Figure 1E), or magenta (Figure 1B), or de mixture of this colors, green with small magenta dots on some calluses (Figure 1I), green with the center becoming magenta (Figure 1A, C and D) and the inverse, and most magenta/pink with some clear green parts (Figure 1F). Pigmentation changes were not found along the entire epidermal surface, but only in specific regions, thus forming these mosaics of colors.

The calli grown for 40 days in the MBI composed of white, green, and magenta spherical-cell agglomerates, and an opaque color (clear green) was also observed in the calli surface (Figure 1F). Under red light, all the

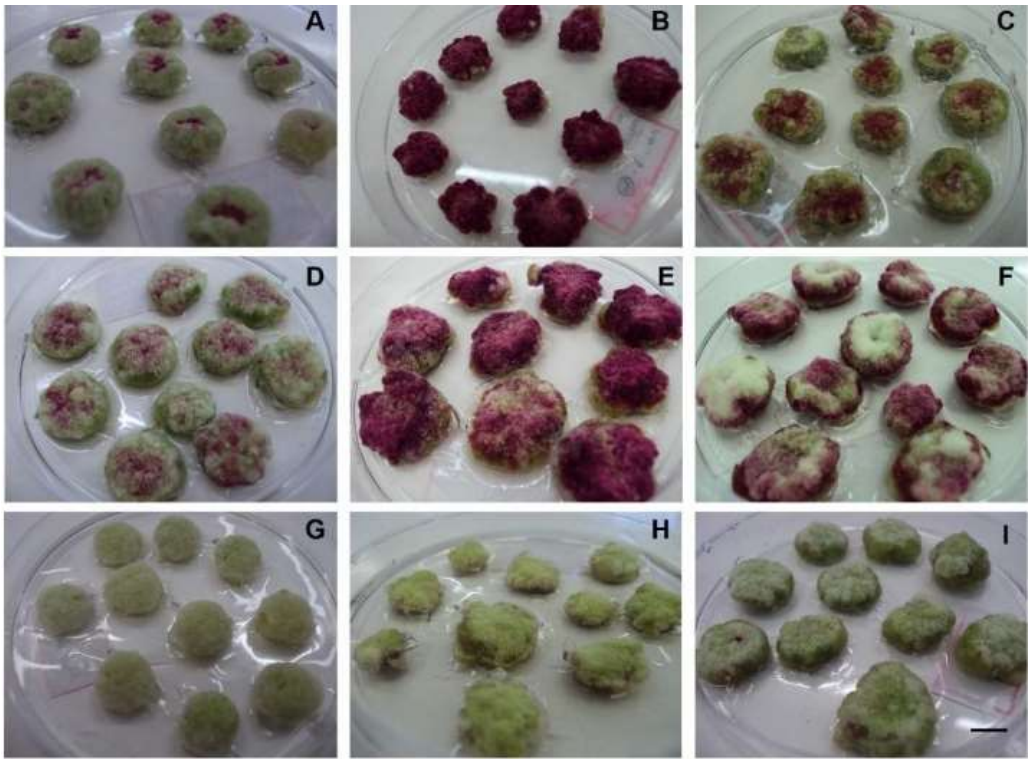

Figure 1. Pigmentation in *Alternanthera brasiliana* callus. Explants from internodes, grown in different culture medium for callus induction (MCI) and after different wavelengths of light, for 40 days in medium for betacyanin induction (MBI). (A) MCI-1: 1 mg L^{-1} KIN and 1 mg L^{-1} 2,4-D + MBI (0.5 mg L^{-1} TDZ and 1 mg L^{-1} NAA) under blue light; (B) MCI-2: 0.75 mg L^{-1} IAA and 1 mg L^{-1} 2,4-D + MBI under blue light; (C) MCI-3: 1 mg L^{-1} BA and 0.5 mg L^{-1} 2,4-D + MBI under blue light; (D) MCI-1 + MBI under white light; (E) MCI-2 + MBI under white light; (F) MCI-3 + MBI under white light; (G) MCI-1 + MBI under red light; (H) MCI-2 + MBI under red light and (I) MCI-3 + MBI under red light. Bar = 1 cm.

experimental plates, with the different combination of phytoregulators showed oxidation of the calli tissue.

As the only source of photosynthesis and a vital environmental factor, light plays an important role in directing photosynthetic biosynthesis and photo-morphogenesis, which are correlated with the phytochemical biosynthesis and accumulation (Bian et al., 2014). Although, in the experiment, all plants were clones, same growth age and initial culture medium, in the first phase of callogenesis induction, different growth regulators were used, which caused the formation of tissues with their inherent characteristics and, allowing different photomorphogenesis and demonstrating these characteristics phenotypically and metabolically by the diverse colorations presented in the callus.

The light quality (spectrum) has the potential to be involved in the shades of betalains seen in *A. brasiliana* calli, where it promoted a great impact on the morphogenesis and metabolism of these structures. The apparent colors of the calli depended on the level of light exposure for the three media combinations used. The explants cultivated in blue and white lights provided a higher number of calli with pigmented dots, partially and fully magenta.

These qualities of light were found as the most visually pigmented treatment, in MCI-2 (Figure 1B and E, for blue and white light, respectively), where the calli were 100% magenta and associated with the presence of betalains. In the red light, however, there was no formation of apparent pigments, barely small spots in some callus (less than 5% magenta) in the MCI-3 (Figure 1I).

The evaluation of color intensity showed the same pattern for color appearance: the best lights for production of pigments are moderate and high intensity white and blue lights. The calli grown in MCI-2 had the highest intensity of magenta color for all light regimes, with moderate to intense coloration being displayed (Figure 2B). The statistical analysis corresponds to the score values as shown in Figure 2.

RCGR and Wc

The effects of light regime are felt throughout the process of plant morphogenesis. Thus, through the analysis of RCGR and the Wc, the differences in the growth pattern

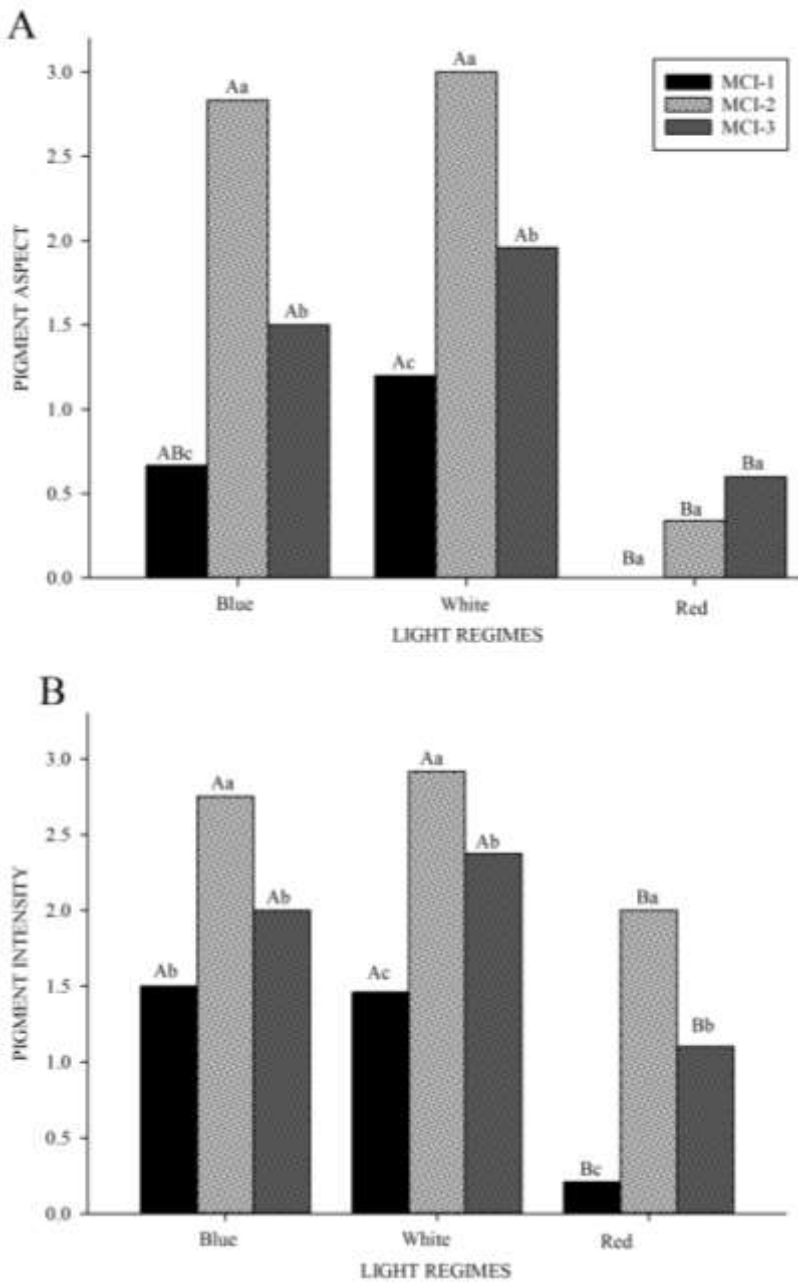

Figure 2. Pigment appearance (A) and pigment intensity (B) in *Alternathera brasiliana* callus after cultivation in Medium for Betacyanin Induction (MBI), under different light regimes (for the media composition, please see Figure 1). The results are represented by the average of four observations with the following scale: zero (absence/lacking of color in the callus), 1 (random pink dots/low color intensity in the callus), 2 (50% of magenta/moderate color intensity in the callus), 3 (100% of magenta/high color intensity in the callus). Different letters indicate significant differences (at $P<0.05$) among the light quality (capital letters) and the three medium studied (small letters).

were clearly visualized in the different light qualities tested.

RCGR in blue and white light exposure the outcomes stood out from the results in red light (Figure 3A). For calli grown on MCI-1, their RCGR did not differ among the three light levels. When grown on MCI-2, the cultivation under white light promoted better growth results in these cells formations. The RCGR in MCI-3 was better

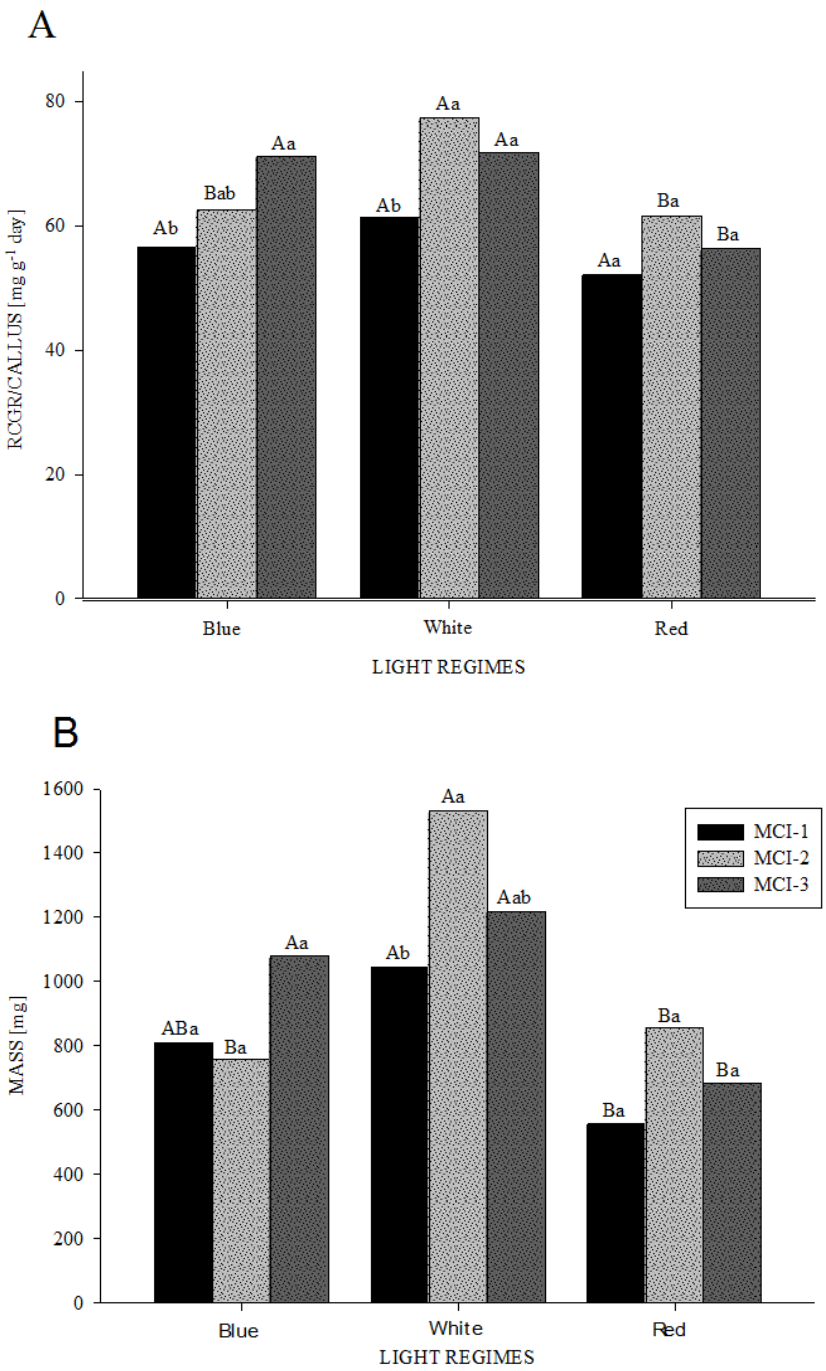

Figure 3. Relative callus growth rate (RCGR) (A) and callus total weight (Wc) (B) of *Alternathera brasiliana* calli, after cultivation in Medium for Betacyanin Induction (MBI), under different light regimes. Different letters indicate significant differences (at $P<0.05$) among the light regimes (capital letters) and the three medium studied (small letters). For the media composition, please see Figure 1.

influenced by white and blue lights, with similar results, found for a daily increase in calli cell mass in these two light qualities. About the different medium in the same light, under white light, the RCGR had better results for MIC-1 and 2; MIC-3 presented the best results in blue light, although, did not differ statistically from the MCI-2 medium cultured in this quality of light. Under red light, however, significant differences were not detected among the different media for calli cultivation. Corresponding to the Wc, the greatest mass accumulation was visualized

Table 1. Betalains[a] (betacianin and betaxanthin) contents in *Alternanthera brasiliana* calli cultivated in Medium for Betacyanin Induction (MBI), under different light qualities.

Analysis	Media	Light regimes		
		White	Blue	Red
Betaxanthin (miraxantin V)	MCI-1	$0.231^{Ba} \pm 0.037$	$1.906^{Ac} \pm 0.676$	$0.914^{Bb} \pm 0.320$
	MCI-2	$0.387^{Ca} \pm 0.118$	$4.663^{Aa} \pm 0.934$	$1.503^{Bab} \pm 0.302$
	MCI-3	$0.339^{Ca} \pm 0.135$	$3.245^{Ab} \pm 0.580$	$1.885^{Ba} \pm 1.450$
Betanin	MCI-1	$0.545^{Aa} \pm 0.195$	$2.029^{Ac} \pm 0.763$	$0.798^{Aa} \pm 0.321$
	MCI-2	$1.835^{Ba} \pm 0.646$	$8.201^{Aa} \pm 3.521$	$1.566^{Ba} \pm 0.471$
	MCI-3	$1.339^{Ba} \pm 0.661$	$5.106^{Ab} \pm 1.186$	$1.867^{Ba} \pm 1.670$
Betanidin	MCI-1	$0.124^{Ab} \pm 0.086$	$0.622^{Ab} \pm 0.296$	$0.126^{Aa} \pm 0.043$
	MCI-2	$4.640^{Aa} \pm 2.106$	$4.350^{Aa} \pm 2.551$	$0.473^{Ba} \pm 0.136$
	MCI-3	$3.225^{Aa} \pm 2.083$	$1.712^{ABb} \pm 0.624$	$0.610^{Ba} \pm 0.369$

[a]Contents (mg 100 g^{-1} fresh weight of *A. brasilana* calli) are means of triplicate determinations (Tukey test at $P < 5\%$) ± standard deviation. Different letters indicate significant differences (at $P<0.05$) among the light quality (capital letters) and the three-medium studied (small letters).

in white light in all combinations of growth regulators tested (Figure 3B). The callus cultivated in MCI-1 showed noteworthy results in white light, but did not differ statistically from the blue light. In the cultures from MCI-2, the white light provided a greater increase in the total cellular mass and those cultured in the MCI-3 medium, had a rise of the mass in the white and blue lights, not statistically different in both. Concerning the correlation between the formation of cell agglomerates and the light quality, under blue and red lights, the final mass is not different among the media, but for the media cultivated in white light, both MCI-2 and 3 yielded calli with a higher final biomass accumulation.

Betaxanthins, betacyanins and total flavonoids quantification

To investigate the additional effects of the light qualities of white, blue, and red levels in the accumulation of secondary metabolites in the *A. brasiliana* calli, the samples were subjected to a quantification analysis of their betalain pigments and of their total flavonoids (both after 40 days of cultivation in MBI).

The spectrophotometric analysis revealed that the blue light was more effective in the induction of betaxanthin biosynthesis (Table 1 and Figure 5A, B, and C) in all the media assayed, with the concentration of this pigment peaking in MCI-2. Under irradiation by red light, however, both MCI-2 and MCI-3 induced a higher accumulation of betaxanthins, whereas the white light did not show any significant differences among the treatments.

It shows the results for betanin, which had the highest yield in the calli extracts grown under blue light and in both MCI-2 and MCI-3 (Table 1). The MCI-1 was an exception for blue light and did not show significant results in the production of this glycosylated betacyanins, similar to the treatment using white light, which was not statistically significant in the media culture used.

The results of the quantification of betanidin in calli are presented in Table 1. The blue light was inductor for this type of pigment production in the MCI-2 and MCI-3. Under blue light, pigment production was higher in the calli cultivated in MCI-2, medium composed by two auxins. For the calli subjected to white light, calli inducted in MCI-2 and MCI-3 had higher biosynthetic production of aglycones. MCI-1 and red-light treatments did not present statistical differences in the treatments.

In the quantification of total flavonoids (Figure 4), all the culture media treatments produced a significant effect independently of the light quality to which it was submitted. The media containing only auxins (MCI-2) and cytokinin combined to auxin (MCI-3), presented the best results as inductors for the biosynthesis of flavonoids in *A. brasiliana* calli.

The estimated Normalized Index of Variation (NIV) helps to visualize the relative effects of the red and blue qualities of light in different culture media. In MCI-1, betaxanthin, betanin, and betanidin indices were higher (0.78, 0.58 and 0.66, respectively) when treated with the blue light rather than that by red light (the latter had values closer to the control, white light). The NIV values for flavonoids in different light qualities did not change compared to those in the control (Figure 5A).

In MCI-2, there is an increase in NIV values for the content of betaxanthin (0.84) and betanin (0.63) under the treatment of blue light. However, neither the blue or red lights increased the content of flavonoids and betanidin (Figure 5B). In MCI-3, the NIV-related content of betaxanthin and betanin enhance under blue light

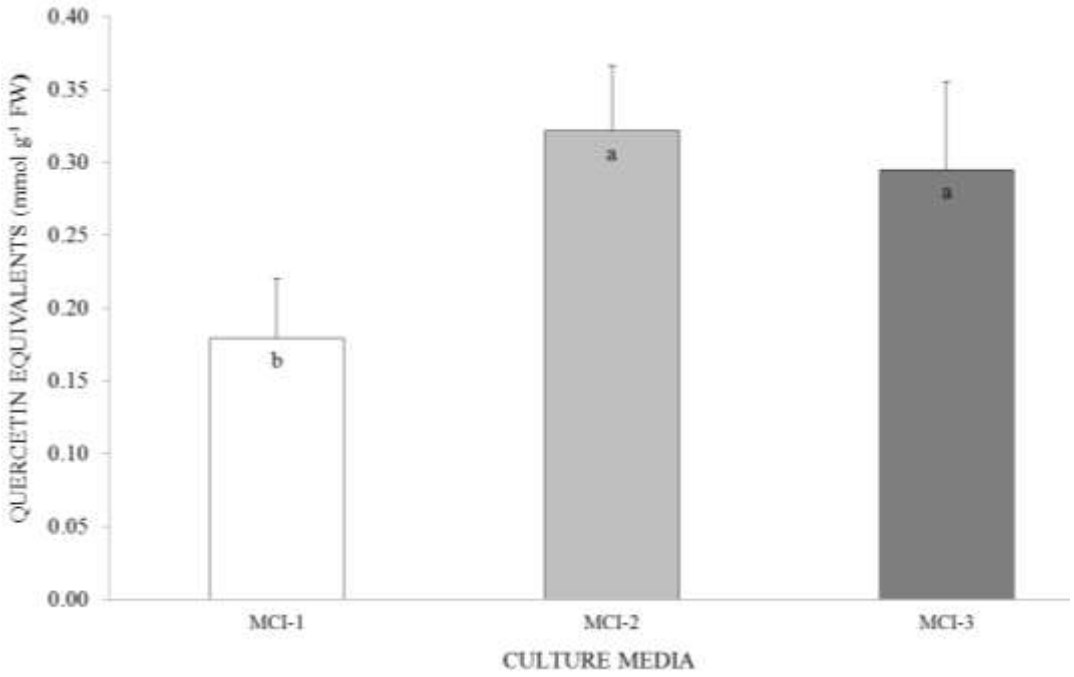

Figure 4. Quantification of total flavonoids in *Alternanthera brasiliana* calli, after Medium for calli induction and cultivation in Medium for Betacyanin Induction (MBI), under different light regimes. For the media composition, please see Figure 1. Contents are means of triplicate determinations ± standard deviation and the average followed by the same letters did not present statistical differences after Turkey´s test *(P*<0.05). Fresh weight (FW).

(0.81 and 0.58, respectively) but not so for contents of flavonoid (-0.013) and betanidin (-0.30). For the last variable, however, both light qualities of blue and red had a negative effect compared to the control (Figure 5B and C).

DISCUSSION

The quality of the light spectrum and the culture medium can influence the morphogenesis process from the entire plant to those grown *in vitro*. Species like *Camptotheca acuminata* had their seedlings studied with different qualities of light demonstrating that the development, leaf area, chloroplast development and photosynthetic efficiency were modified by the use of red light rather than blue or yellow light (Yu et al., 2017). In strawberry (*Fragaria* × *ananassa*), the total content of anthocyanins increased by the use of red and yellow plastic films, suggesting the activation of enzymes or transcription factors related to the flavonoid pathway (Miao et al., 2016).

As reported in the methods, the period of darkness at the beginning of calli induction were required for the formation of the cell masses and the reduction of phenolic compounds, which can affect the explants; this was specified by Tan et al. (2010). The changes in the calli pigments and its intensity were visualized in the first week after exposing the plates in an environment treated by different kinds of light (visual observations, data not shown), firstly in white light. Reports have demonstrated that betalains biosynthesis occurs between the sixth and fifteenth days, but is most intense towards the ninth day (Radfar et al., 2012).

The development of different lineages with colored cells in plant tissue cultures depends on specific gene sequences in the calli induction and these individual phenotypes, once established, can be perpetuated and maintained in the culture medium (Girod and Zyrd, 1991). A determining factor for the establishment and stability of these lineages is the composition of the culture medium for *A. brasiliana* calli. The MS medium containing IAA (0.75 mg L^{-1}) and 2,4-D (1 mg L^{-1}) exposed to blue and white lights have been highlighted as the strongest inductors for production and intensification of the pigments. In studies that used *Zaleya decandra* calli grown under white light (Radfar et al., 2012), the highest pigments intensity was seen when the samples were cultured on the MS-plus-TDZ (2 mg L^{-1}) and 2,4-D (1 mg L^{-1}). However, it is not entirely clear whether the intense pigmentation is due to an increase of metabolic activity in individual cells, or simply an increase in the number of cells able to produce betalains (Kishima et al., 1991).

Changes in the combinations of the plant growth

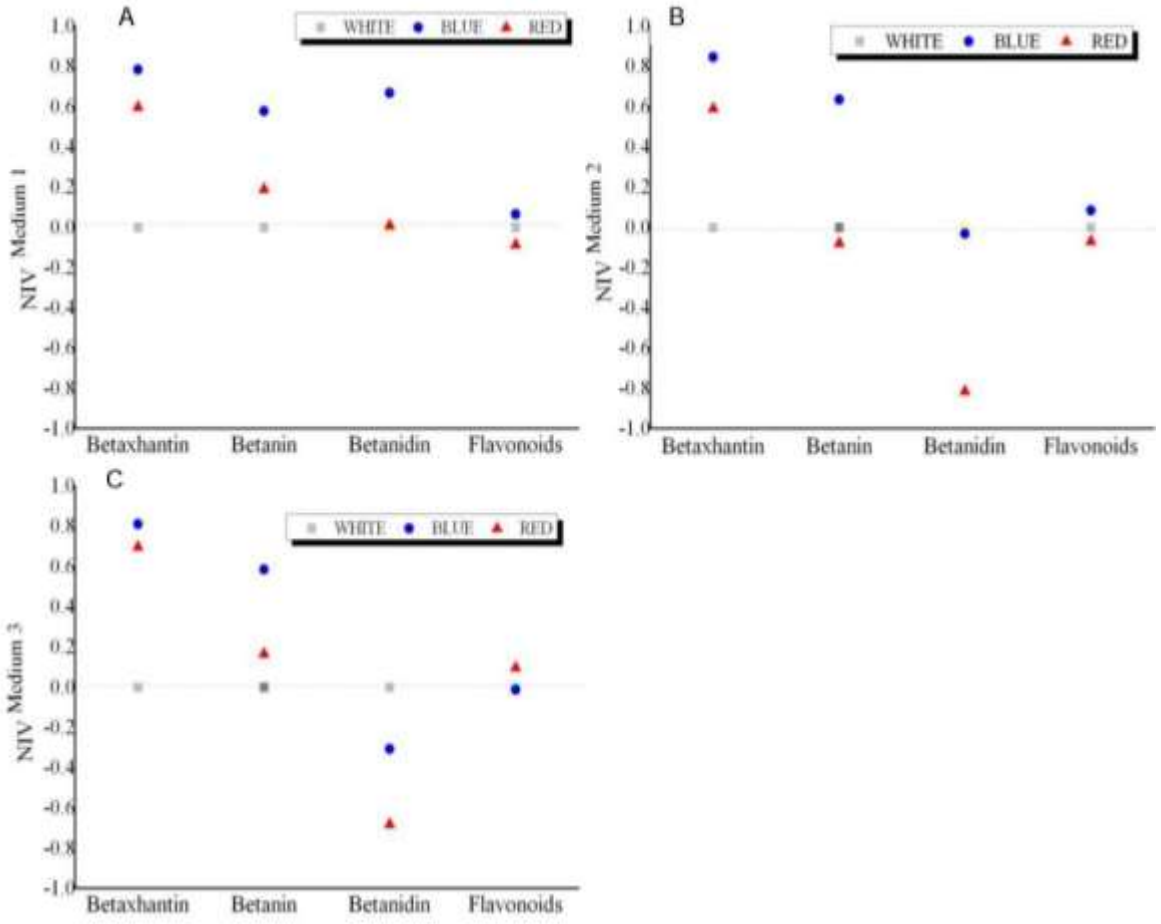

Figure 5. Normalized Index of Variation for betaxanthin, betanin, betanidin and flavonoids in *A. brasiliana* callus growth in Medium for calli induction and cultivation in Medium for Betacyanin Induction (MBI), under different light qualities. For the media composition, please see Figure 1. The dotted horizontal line shows the normalized values of the treatment with white light.

regulators in a culture medium can be used to modulate the frequency and direction of the inter-conversion events that result in chimeric phenotypes, which are calli with only one-color pattern and after to transferring them to a medium with low concentration of 2,4-D, sectors with another coloration arise (Leathers et al., 1992). The speed of this response, which may be 1-2 cell generations, indicates that the transformation of the phenotype, induced by hormones, it is associated with cellular DNA replication and therefore susceptible to a cell proliferation process (Girod and Zryd, 1991). In other words, depending on the effect induced by the phytohormones, a phenotype can change the color and shape of the cells made of it.

The RCGR and Wc of *A. brasiliana* callus showed the best results under white and blue lights, agreeing with results reported for another species as *Suaeda salsa* (Zhao et al., 2010), which had a RCGR markedly higher in calli grown under white light. The calli grown on medium containing IAA and 2,4-D (MCI- 2) and medium with BA and 2,4-D (MCI-3) had higher rates of daily growth, as well as higher final mass accumulations. Studies done with *Portulaca* species calli (Noda and Adachi, 2000) showed the highest growth rate in calli cultured on a MS medium supplemented with 5 to 10 μM of 2,4-D, with identical growth rates in different concentrations of auxin. Also, calli growth is higher when auxins are incorporated in the medium than when compared with that supplemented by cytokinins (Lee et al., 2011; Abu-romman and Suwwan, 2013).

The accumulation of betacyanins when the plates were submitted to cultivation under darkness indicates that the light exposure is not a prerequisite for the formation of betacyanins in some plant species, but rather a powerful stimulant for their biosynthesis (Leathers et al., 1992). This light-induced mechanism of biosynthesis begins when the light signal from the phytochrome or cryptochrome passes through multiple signaling intermediates, which then regulates a transcription factor that can control the expression of genes encoding key

enzymes such as tyrosinase, DOPA oxidase, and glycosyl transferases, thereby triggering their post-translational modification, a process of fundamental importance in the formation of this pigment (Zhao et al., 2010).

Exposure of *Amaranthus tricolor* (Elliott, 1979) and *Celosia plumose* (Nicola et al., 1974) seedlings to red- and white-light lamps increased their betacyanins and betaxanthins production. In *A. brasiliana,* the highest biosynthesis of betaxanthins and glycosylated betacyanins was visualized in MCI-2 medium after exposure to blue light. These results corresponded to an approximately 12- and 4.5-fold increase in productivity when compared with the white light that is commonly used in tissue culture. Similar results were reported for cell suspensions of *Chenopodium rubrum*, for which an increase of 30% in amaranthin levels and 10% of betanin when cultivated under blue light (Berlin et al., 1986).

Studies about the synergistic effect between kinetin and light in *A. tricolor* seedlings (Bianco-Colomas and Hugues, 1990) showed extremely positive results, with a high accumulation of betacyanins. By contrast, the results showed that the highest betacyanin concentration arose from betacyanins aglycones in a medium composed of auxin; these results showed that exposure to blue and white lights induced approximately 10 times more aglycones than under red light. It seems, therefore, that the pigmentation response generated by the luminous environment is an intrinsic feature of the cells themselves and related to the species to which they belong (Kishima et al., 1991).

The quantification of total flavonoids revealed higher amounts in the MCI-2 and 3, agreeing with results obtained using *Morus alba* in which the auxins strongly induced the production of flavonoids in calli from adventitious roots cultured on MS medium supplemented with 5 mg L^{-1} of IAA (Lee et al., 2011).

Interestingly, in studies using cell cultures of *Centella asiatica*, adding 2,4-D and kinetin to the culture media stimulated the production of flavonoids as quercetin, kaempferol, luteolin and rutin (Tan et al., 2010). It is evident then, in a general picture, that the MCI-2 promoted the highest biosynthesis of metabolites, regardless of the level in light quality, when composed of a natural and a synthetic auxin (IAA, 0.75 mg L^{-1} and 2,4-D, 1 mg L^{-1}), respectively. Both auxins play an important role in the induction of calli and various auxins can have different effects, although the synthetic auxins in many cases are more effective than the natural ones (Baskaran et al., 2014).

According to the results, the best medium for calli with a greater amount of mass and RCGR is the MCI-2, supplemented with both IAA and 2,4-D before inoculation on the MBI and cultivation in white light was ideal for *A. brasiliana* callus.

The MCI-2 was the best also for the production of the metabolites of interest in this study; so, for production of quercetin-type flavonoids, white light has an essential importance. In the production of betacyanin aglycone-types, the white and blue qualities of lights are both inductive of these pigments. However, for the biosynthesis of a greater amount of betacyanin glycosylated-types and for betaxanthins in *A. brasiliana* calli, it is indicated that the induction phase of the betalains is carried out under blue light, reaching a greater quantity of pigments in this quality of light. In these experiments, the red light was ineffective at inducing pigmentation in the calli of this plant species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Simple sequence repeat (SSR) markers linked to drought tolerant traits in selected Sudanese rice (*Oryza sativa* L.*)* genotypes

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Rice growth and productivity are affected by biotic and abiotic stresses; key among them being drought. Conventional breeding for drought tolerance is slowed down by the complex nature of mechanisms underlying this stress although molecular markers offer a promising approach. Plant exposure to drought stress leads to a significant effect in chlorophyll content and reduction in photosynthesis. In this study, 23 Sudanese rice (*Oryza sativa* L.) genotypes under greenhouse conditions for drought tolerance were examined. The study focus on tracking changes in chlorophyll content under stress (by withholding water and later rewatering) and further screened them for DNA polymorphisms using simple sequence repeat (SSR) markers. The results showed that genotypes IR11A306, IRRI 154, NERCA 6, IR12N 240, NERICA 4, Wakra and IRRI 150 exhibited high drought tolerance based on the assay. After dehydration, IR11A306 recorded the highest increment in total chlorophyll while IR11A483 showed the highest reduction followed by NERICA 15, IR11 N121, IRRI 168, NERICA 7, NERICA 1, NERICA 14 and Nipponbare. When plants were rehydrated and total chlorophyll measured, the highest increase and best recovery were observed in IR74371-70-1-1 followed by IRRI 168, IRRI 147, Nipponbare, Kosti 2, IAC 165 and Umgar. Genotype IR11A306 showed the least reduction in chlorophyll followed by NERICA 15, IRRI 150, IRRI 122, IR12N 240, IRRI 154, NERICA 16, NERICA 4 and Wakra. Eighteen out of the 19 primers tested showed amplification of the SSR markers generating 569 alleles that ranged between 13 and 113 alleles per marker. These alleles further produced polymorphism information content (PIC) values of 0.51 to 0.99 per marker. The assay helped select genotypes that showed a steady recovery of chlorophyll content following drought stress while the markers studied could be useful for future molecular breeding for drought tolerance in rice.

Key words: Chlorophyll, drought tolerance, polymorphism, rice, simple sequence repeats (SSR), sudanese genotypes.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food crops providing 27% of the world nutritional energy

and 20% of overall nutritional protein (Muench et al., 1998; Bashir et al., 2007). It is cultivated under diverse ecologies ranging from irrigated to rain-fed uplands, rain-fed lowland and deep water. Irrigated rice is cultivated on 55% of the world's production area and accounts for about 75% of total rice production. Furthermore, over 3 billion people in the world depend on rice for food (Awasthi and Lal, 2014). In Africa, Sudan has an estimated rice production area of more than 300,000 ha and if this area is properly utilized, it would suffice the local consumption demand and hence fill the gap with non-course food grain. Rice production in Sudan was introduced in the Gazira scheme by the technical assistance of China in 1973 and is manly practiced in the southern states. During this period, 12,000 ha were cultivated under irrigation with yields of between 3.5 and 7.6 ton/ha attained. A few years ago, it was reported that, no breakthrough with regards to increasing the cultivated area and improving new varieties has been achieved (Mahgoub, 2014). Being in an arid and desert ecological zone, Sudan is one of the most vulnerable countries to climate change owing to a high climatic variability and low development (Elasha et al., 2005). Drought has been considered one of the major causes of food insecurity in Sudan (Mahgoub, 2014) and in many parts of the country, this was been exacerbated by decreasing annual rainfall over the past 60 years (Nimir and Elgizouli, 2011).

Growth and productivity of rice is adversely affected by various biotic and abiotic stress factors key among them being drought (Ndjiondjop et al., 2010; Singh et al., 2012). Drought conditions lead to a reduction in plant growth by affecting various physiological and biochemical processes such as photosynthesis, respiration, translocation, ion uptake, carbohydrate metabolism, nutrient metabolism and growth promoter activities (Farooq et al., 2008). Drought stress leads to a reduction in leaf area, cell size and intercellular volume (Ndjiondjop et al., 2010). Furthermore, it has been demonstrated that exposure of plants to drought leads to a significant effect in chlorophyll contents as a result of the reduction in leaf growth (Chutia and Borah, 2012). Most of the cultivated rice varieties are susceptible to drought therefore necessitating the need for continued improvement (Uphoff et al., 2015). In attempts to overcome the drought problem, researchers in various improvement programs have used molecular markers to identify germplasm with traits related to drought tolerance (Afiukwa et al., 2016).

Development of molecular markers and their use for genetic dissection of agronomical important traits has been identified as a powerful tool for studying complex plant traits such as drought tolerance (Suji et al., 2011). Particularly, DNA-based molecular markers have been reliably used with availability of a large number of polymorphic markers enabling precise classification of the cultivars (Sonia, 2013). Improvement of rice for drought tolerance using conventional breeding methods is slow due to geographical differences and the variations of seasons in drought timing and severity, the complex nature of drought tolerance traits and the difficulty in selection of combinations of traits (Courtois et al., 2003). Other factors that have slowed down this process include low heritability, multiple gene control as well as genotype and environmental interactions (Cattivelli et al., 2008). The uses of molecular markers to select accessions possessing genes and genomic regions that control target traits can fast-track the progress in breeding drought tolerant rice. This is because molecular markers are consistently transmitted from generation to generation and are not subject to environmental influences (Afiukwa et al., 2016). Studies using molecular markers have reported success in identification of QTLs underlying various drought tolerance traits in rice chromosomes. For instance, Vasant (2012) showed that 12 SSR markers are strongly associated with root traits under drought while 14 SSRs show a significant association with yield and its components under drought. Furthermore, other molecular markers associated with drought-related traits have also been reported thereby affirming the usefulness of these markers in the molecular breeding of rice for improved drought tolerance (Afiukwa et al., 2016). Several molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphisms (SNPs) are presently available to assess the variability and diversity at molecular level and have been used to enhance traditional breeding programs to improve rice crop (Sonia, 2013). The SSR markers were identified as the system of choice for genetic analysis in rice because of their efficiency, abundance in the rice genome, high level of polymorphism and high but simple reproducible assays that are reliable (Singh et al., 2010). This study therefore aimed to determine the SSR markers linked to drought tolerance traits and their association with phenotypic traits in rice genotypes cultivated in Sudan. The study also assayed for changes in total chlorophyll content following drought stress by withholding water from the plants and later re-watering them. Data obtained here is expected to contribute towards marker assisted breeding for drought tolerance for sustainable rice production under the current climate changing conditions.

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Table 1. List of rice genotypes used in the study.

Name	Origin (Country, Group)	Ecosystem	level of drought tolerance
NERICA 6	Sierra Leone	Upland	Drought tolerant
NERICA 16	Sierra Leone	Upland	Drought tolerant
NERICA 15	Sierra Leone	Upland	Drought tolerant
NERICA 14	Sierra Leone	Upland	Drought tolerant
NERICA 4	Sierra Leone	Upland	Drought tolerant
Wakra	Indonesia	Upland	Drought tolerant
Kosti 2	WARDA	Upland	Drought tolerant
Kosti 1	WARDA	Upland	Drought tolerant
IRRI 150	Philippines	Undefined	Undefined
IR11A306	IRSEA	Undefined	Undefined
IR11A483	IRSEA	Undefined	Undefined
IRRI 147	Philippines	Undefined	Undefined
IRRI 122	Philippines	Undefined	Undefined
NERICA 1	Sierra Leone	Upland	Drought tolerant
IR12N240	IRSEA	Undefined	Undefined
IR74371-70-1-1	India	Undefined	Drought tolerant
IR11N121	IRSEA	Undefined	Undefined
IRRI 154	Philippines	Undefined	Undefined
IRRI 168	Philippines	Undefined	Undefined
Umgar	China	Upland	Drought tolerant
Nipponbare	Japan	Upland	Undefined
IAC 165	Brazil	Upland	Undefined
NERICA 7	Sierra Leone	Upland	Drought tolerant

MATERIALS AND METHODS

Plant materials

Seeds of twenty three (23) *Oryza sativa* L. genotypes grown in Sudan with varying degree of tolerance to drought were obtained from Biotechnology and Biosafety Research Center (BBRC), Agricultural Research Corporation, Sudan and used. These included 11 upland drought tolerant genotypes (NERICA 4, NERICA 6, NERICA 14, NERICA 15, NERICA 16, NERICA 1, NERICA 7, Umgar¸ Kosti 1, Kosti 2 and Wakra), ten genotypes (IRRI 150, IR 11A306, IR 11A483, IRRI 147, IRRI 122, IR 12N240, IR 74371-70-1-1, IR11N121, IRRI 154 and IRRI 168) still under research in Sudan and two genotypes (IAC 165 and Nipponbare) from Kenyatta University (KU), Kenya. Genotype description is presented in Table 1.

Determination of chlorophyll content from rice

Chlorophyll content analysis was conducted on rice leaves grown in the greenhouse at the Plant Transformation Laboratory, Kenyatta University, Kenya. Rice seeds were directly sowed in plastic pots (size 10*10*5 cm) containing garden soil placed in buckets containing water for underwatering. The greenhouse conditions were: 12 h light/12 h dark photoperiod; 28ºC day and 24ºC night temperature and 60% of humidity. Five plants were used for each genotype with three replicates. Pots were randomized and irrigated after every five days. At the 3 to 4 leaf stage (corresponding to around 3 weeks after sowing), the plants were irrigated constantly until 60 days followed by the first chlorophyll extraction according to Botstein et al. (1980). Five leaf discs were punched from rice plants using a paper punch and ground to a fine powder under liquid nitrogen. Total chlorophyll was then extracted using acetone with a 10 min centrifugation step. The chlorophyll was quantified using a spectrophotometer (Spectrometre UV- Visible- UV- 3100 PC- VWR) under 660 nm wavelength. Three spectrophotometer readings were taken and an average calculated for each genotype. The plants were exposed to drought stress conditions by draining water from the buckets and placing back the plastic pots. The plants were maintained under this condition for seven days after which a second chlorophyll extraction was done. Plants were then put under the normal condition by rewatering the buckets and a 3rd chlorophyll extraction was done seven days later.

Screening rice genotypes using SSR markers

Genomic DNA was isolated from fresh three week-old rice leaf samples grown in the greenhouse using a DNA extraction method described by Murray and Thompson (1980). Quality of the extracted DNA was determined using agarose gel electrophoresis and quantified using a nanodrop. Eighteen (18) SSR primers previously reported to have association with drought tolerance traits in rice (Afiukwa et al., 2016) were used for PCR amplification to test any polymorphisms among the rice accessions under this study. Primer description is shown in Table 2. PCR was done in 25 µl reaction mixture comprising 1X PCR master mix (New England Biolabs), 0.25 µM of each primer (forward and reverse), 10 ng/µl of template DNA and deionised water. The reactions were carried out in a thermocycler (Eppendorf Inc.) under the following conditions; an initial heating step of 95ºC for 3 min (denaturation) followed by 35

Table 2. List of SSR markers used to in the current study.

Primer name	Primer sequence	SSR motif	Chr. Num.	Size of bands	Sources
RM38	F: ACGAGCTCTCGATCAGCCTA R: TCGGTCTCCATGTCCCAC	(GA)16	8	250	Srividhya et al., 2011
RM252	F: TTCGCTGACGTGATAGGTTG R: ATGACTTGATCCCGAGAACG	(CT)19	4	216	McCouch et al., 2002
RM170	F: TCGCGCTTCTTCCTCGTCGACG R: CCCGCTTGCAGAGGAAGCAGCC	(CCT)7	6	121	Yue et al., 2005
RM318	F: GTACGGAAAACATGGTAGGAAG R: TCGAGGGAAGGATCTGGTC	(GT)15	2	140	Srividhya et al., 2011
RM279	F:GCGGGAGAGGGATCTCCT R: GGCTAGGAGTTAACCTCGCG	(GA)16	2	174	Ordonez et al., 2010
RM7390	F: CTGGTTAACGTGAGAGCTCG R: GCAGATCAATTGGGGAGTAC	(GATA)8	9	140	McCouch et al., 2002
RM432	F: TTCTGTCTCACGCTGGATTG R: AGCTGCGTACGTGATGAATG	(CATC)9	7	187	Vikram et al., 2011
RM5367	F: AGTACCTCTCACTCGCCTGC R: TGTCAGCTGTGAGTGAAGTCG	(TC)13	12	185	McCouch et al., 2002
RM5423	F: ATCCCACTTGCAGACGTAGG R: ACAGCAGCAAGGTGCCTC	(TC)16	1	202	McCouch et al., 2002
RM5850	F: TTAGGTGTGTGAGCGTGGC R: ATACACAGATGACGCACACG	(ATA)27	6	181	McCouch et al., 2002
RM36	F: CAACTATGCACCATTGTCGC R: GTACTCCACAAGACCGTACC	(GA)23	3	192	Brondani et al., 2002
RM3558	F: ACGAGAGATCTTCTTTGCAG R: CCTCTATTTATGCCTCTACGC	(GA)12	4	161	McCouch et al., 2002
RM517	F: GGCTTACTGGCTTCGATTTG R: CGTCTCCTTTGGTTAGTGCC	(CT)15	3	266	Lu et al., 2005
RM6130	F: GGCAGAGAGAGCTGCATCTC R: GACGACGACGAACCCAAC	(CGC)8	4	116	McCouch et al., 2002
RM583	F: AGATCCATCCCTGTGGAGAG R: GCGAACTCGCGTTGTAATC	(CTT)20	1	192	Vikram et al., 2011
RM1141	F: TGCATTGCAGAGAGCTCTTG R: CAGGGCTTTGTAAGAGGTGC	(AG)12	1	100	McCouch et al., 2002
RM260	F: ACTCCACTATGACCCAGAG R: GAACAATCCCTTCTACGATCG	(CT)34	12	111	McCouch et al., 2002
RM525	F: GGCCCGTCCAAGAAATATTG R: CGGTGAGACAGAATCCTTACG	(AAG)12	2	131	McCouch et al., 2002
RM331	F: GAACCAGAGGACAAAAATGC R: CATCATACATTTGCAGCCAG	[(CTT)4GTT]2(CTT)11	8	176	Srividhya et al., 2011

cycles of 94ºC for 30 s, annealing at 55ºC for 30 s and an extension period of 68ºC for 30 s. A final extension period of 68ºC for 5 min was also included. The amplified PCR products were resolved on a 2% agarose gel after staining with SYBR green and run at 100 volts for 1 h alongside a 50 bp DNA ladder. The gels were visualized under UV light from a transilluminator and documented using a digital camera. The amplified bands were scored for each SSR marker, generating a binary data matrix of 1 (presence) and 0 (absence) for each primer. This information was then used to determine the number of alleles and the Polymorphism Information Content (PIC) by using the formula described by Botstein et al. (1980) as follows:

$$PIC_j = 1-\sum_{i=1}^{n} P_i^2-\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2 P_i^2 P_j^2$$

Where, P_i and P_j are the frequencies of the i^{th} and j^{th} alleles of a given marker, respectively, n = number of different alleles

Statistical analysis

Analysis of variance (ANOVA) was performed to compare

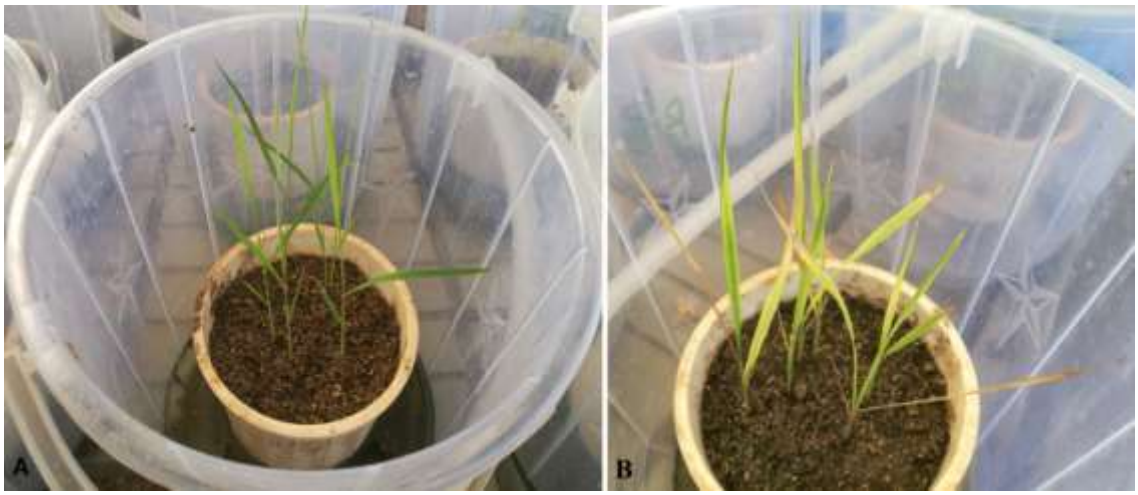

Figure 1. Plants of Kosti 1 genotype in the greenhouse before (A) and after (B) exposure to drought stress by withholding water for 7 days.

chlorophyll contents among genotypes under the imposed drought conditions using Statistical analysis software (SAS) version 9.2. A Tukey's HSD test at 95% confidence interval was used for mean separations and the data presented as means with their respective standard errors. The SSR marker amplification data (1 and 0) were used to determine population genetic structure and presented as a dendrogram generated by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on Jaccard's dissimilarity coefficient in the Dissimilarity Analysis and Representation (DARwin) software version 6. Marker-trait association analysis was done by physically comparing the pattern of the SSR markers clustered from the genotypes with the percentage change in the total chlorophyll content as a result of drought stress.

RESULTS

Effect of drought on rice chlorophyll content

Withholding water for seven days resulted in a marked effect on total chlorophyll content in all rice genotypes under this study. The effect was first noted in the color change in the leaves with those under stress turning yellow compared to plants growing under normal conditions (Figure 1). Total chlorophyll content was significantly affected despite all genotypes responding differently to the drought stress treatment. Under normal watering conditions (control) (Figure 2), NERICA 7 showed the highest chlorophyll content followed by significantly lower levels ($p \leq 0.05$) in NERICA 15, Kosti 1, NERICA 1, NERICA 14, IAC 165, IRRI N 121 and Wakra. Genotype IRRI 154 recorded the lowest chlorophyll followed by Nipponbare, IR12N240, IR11A483, Kosti 2, IR74371-70-1-1 and IR11A306. After dehydration (Figure 3), IR11A306 recorded the highest increment in total chlorophyll followed by NERICA 4, Kosti 1, Wakra, NERICA 6, IR12N240, IAC 165 and IRRI 150, while IR11A483 showed the highest reduction followed by Nipponbare, Kosti 2, IR74371-70-1-1, IRRIN121, IRRI 168, NERICA 7, NERICA 15 and NERICA 1. When plants were rehydrated and total chlorophyll measured (Figure 4), the highest increase and best recovery were observed in IAC 16 followed by Kosti 1, IR 74371-70-1-1, IRRI 147, NERICA 6, Umgar and IRRI 168, while NERICA 15 showed the least reduction in chlorophyll levels followed by IR11A483, IR 11A 306, IRRI 122, IRRI 150, IR12N240 and IRRI 154.

All the genotypes recorded different values of change in total chlorophyll (Table 3). At the end of the dehydration period, genotype IR 11A 306 recorded the highest increase in total chlorophyll followed by IRRI 154, NERICA 6, IR12N240, NERICA 4, Wakra and IRRI 150, while IR11A483 showed the highest reduction followed by Nerica 15, IR11N121, IRRI 168, NERICA 7, NERICA 1, NERICA 14 and Nipponbare. After rehydration, the highest increase and best recovery were observed in IR74371-70-1-1 followed by IRRI 168, IRRI 147, Nipponbare, Kosti 2, IAC 165 and Umgar. Genotype IR11A306 showed the least reduction in chlorophyll levels followed by NERICA 15, IRRI 150, IRRI 122, IR12N240, IRRI 154, NERICA 16, NERICA 4 and Wakra. In general, NERICA 4 and Wakra performed best under both conditions (before and during dehydration) but their chlorophyll contents declined after rehydration. NERICA 6, IRRI 154 and IR11A306 showed a weak performance in normal conditions and after rehydration, but a good performance during the dehydration period.

Population structure of rice genotypes and SSR polymorphism

For determination of the levels of genetic diversity in the population under the current study, a total of 18 out of the

Table 3. Percentage change in total chlorophyll contents following dehydration and rehydration in rice genotypes**.**

S/N	Genotype	% Chlorophyll change after dehydration	Rank	% Chlorophyll change after rehydration	Rank
1	IAC 165	-19.41	9	7.31	6
2	IR 11A 306	187.12	1	-97.03	23
3	IR 11A 483	-82.88	23	-22.98	8
4	IR 12N 240	57.33	4	-83.52	18
5	IR 74371-70-1-1	-46.51	12	257.54	1
6	IRRI 150	2.77	7	-92.17	21
7	IRRI 122	-26.72	10	-91.03	20
8	IRRI 147	-38.22	11	43.39	3
9	IRRI 154	150.53	2	-83.36	18
10	IRRI 168	-73.87	20	63.93	2
11	IRRI N 121	-77.61	21	-63.56	12
12	KOSTI 1	-1.48	8	-26.64	9
13	KOSTI 2	-57.43	15	7.89	5
14	NERICA 1	-61.71	18	-65.39	13
15	NERICA 14	-59.69	17	-68.54	14
16	NERICA 15	-77.98	22	-96.17	22
17	NERICA 16	-51.33	14	-79.81	17
18	NERICA 4	16.79	5	-78.029	16
19	NERICA 6	69.28	3	-51.96	11
20	NERICA 7	-65.89	19	-28.31	10
21	NIPPONBARE	-59.32	16	14.18	4
22	UMGAR	-48.54	13	2.67	7
23	WAKRA	6.89	6	-69.46	15

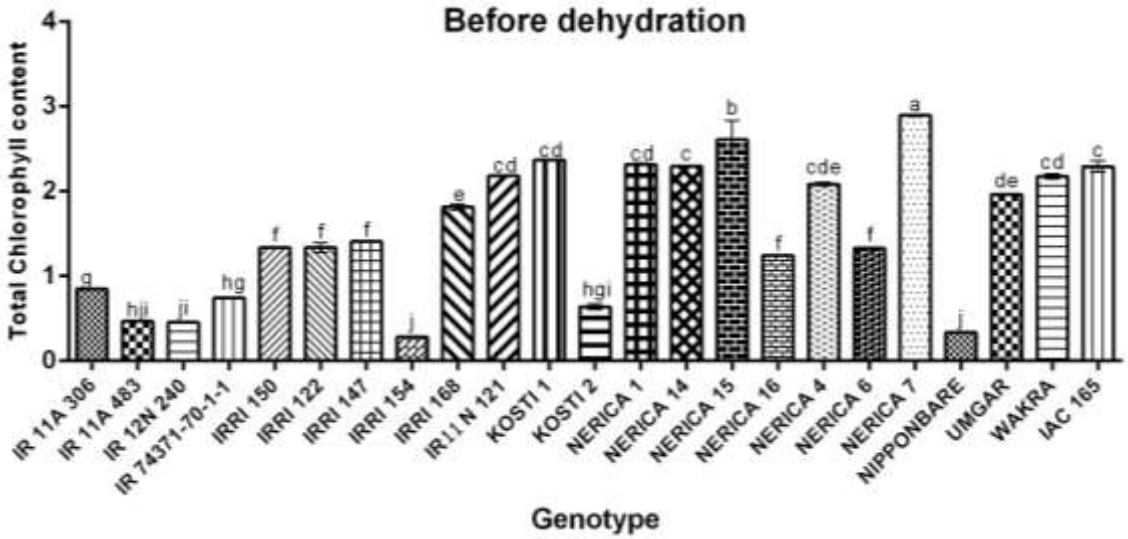

Figure 2. Total chlorophyll content in rice leaves after 2 months of growth under normal conditions. Genotypes (means) with the same letter are not significantly different.

19 SSR markers successfully amplified and representative gel images of the results are presented in Figure 5. Information on allele frequency, allele number and PIC are summarized in Table 4. The 18 SSR primers resulted in a total of 569 alleles with 13 to 113 alleles per primer at an average of 31.7. The PIC values ranged from 0.51 to 0.99 with a mean value of 0.88. Construction of a dendrogram of the 23 rice genotypes using UPGMA

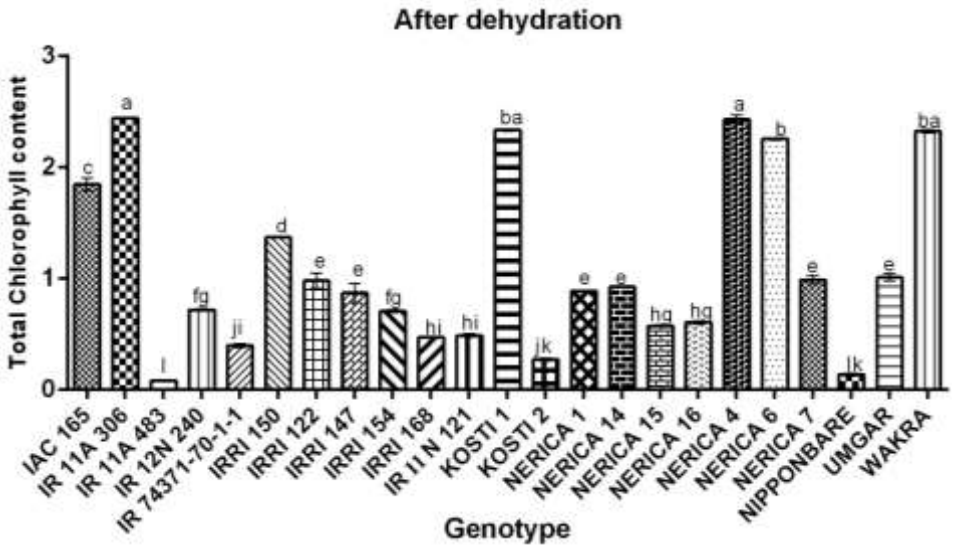

Figure 3. Total chlorophyll content in rice leaves 7 days after dehydration. Genotypes (means) with the same letter are not significantly different.

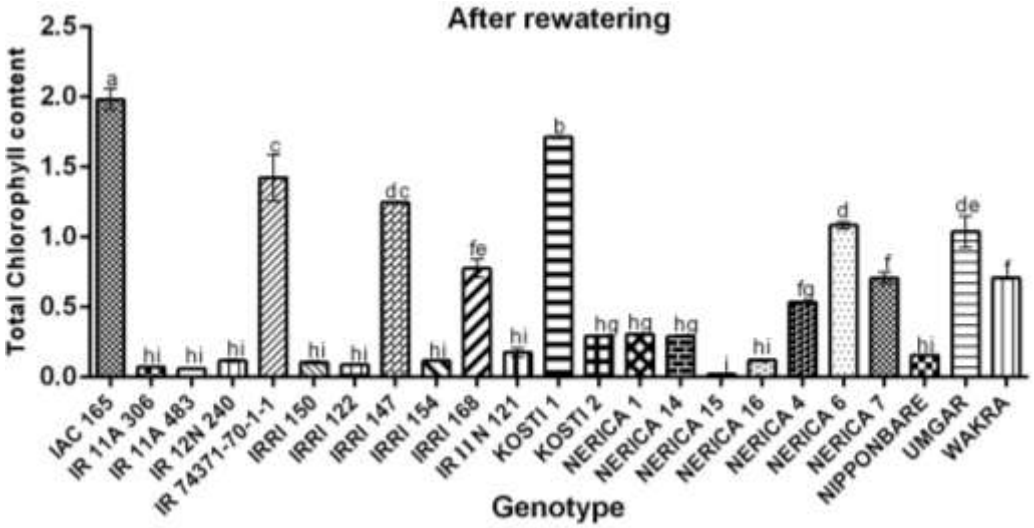

Figure 4. Total chlorophyll content after 7 days of rewatering. Genotypes (means) with the same letter are not significantly different.

method clustered the genotypes into 3 major clusters (Figure 6). Cluster 1 had 2 distinct sub-groups with all the members in this sub-group originating from the upland ecosystem. The genotypes that clustered in this group were Kosti 2, NERICA 14, Kosti 1, Wakra, NERICA 4, NERICA 15, NERICA6, NERICA 7, NERICA 16, IAC 165 and NERICA 1, while sub-group 2 comprised Nipponbare, Umgar and IR12N240. The second cluster comprised lowland rice genotypes and these were further grouped into two sub-group; sub-group 1 that had IR11A306, IRRI 150, IR11A483, IRRI 147, IRRI 122 and IR 74371-70-1-1 and sub-group 2 that had IRRI 154 and IR11N121. A lowland IRRI 168 genotype formed cluster 3.

Marker- trait association under drought stress analysis

The above analysis was used to manually compare the pattern in which the SSR markers grouped the genotypes and the change in total chlorophyll content in each genotype. It was found that none of the markers clearly

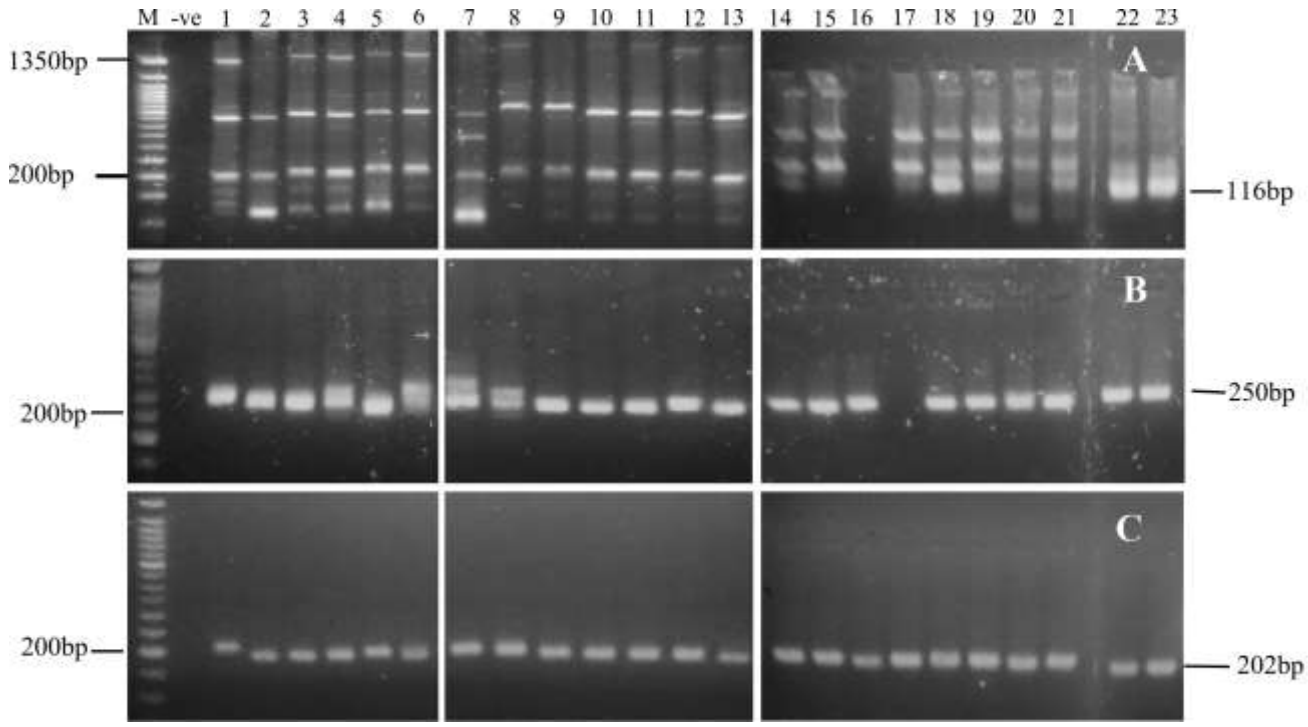

Figure 5. Representative gels of fragments amplified by PCR targeting SSR markers common in size to rice. M -50 bp ladder; -ve= negative control; numbers 1- 23 represent each of the rice varieties; A = RM6130; B = RM 38; C = RM5423.

Table 4. Genetic diversity indices detected in rice genotypes by SSR markers.

SSR primer	Allele frequency	Allele number	PIC
RM38	0.96	27	0.95
RM252	1	27	0.99
RM170	1	33	0.99
RM318	1	37	0.99
RM279	0.74	28	0.73
RM7390	0.52	29	0.51
RM432	0.74	57	0.73
RM5367	1	23	0.99
RM5423	0.96	22	0.95
RM5850	0.57	13	0.56
RM36	1	26	0.99
RM3558	1	23	0.99
RM517	1	24	0.99
RM6130	0.96	113	0.95
RM583	1	23	0.99
RM1141	0.96	22	0.95
RM260	0.61	15	0.6
RM525	1	27	0.99
Mean	0.89	31.7	0.88

grouped the genotypes according to the patterns of change in total chlorophyll content. Although the majority of cluster 1 (Figure 6) are known as upland, only Nerica 6, Nerica 4 and Wakra showed an increase in total

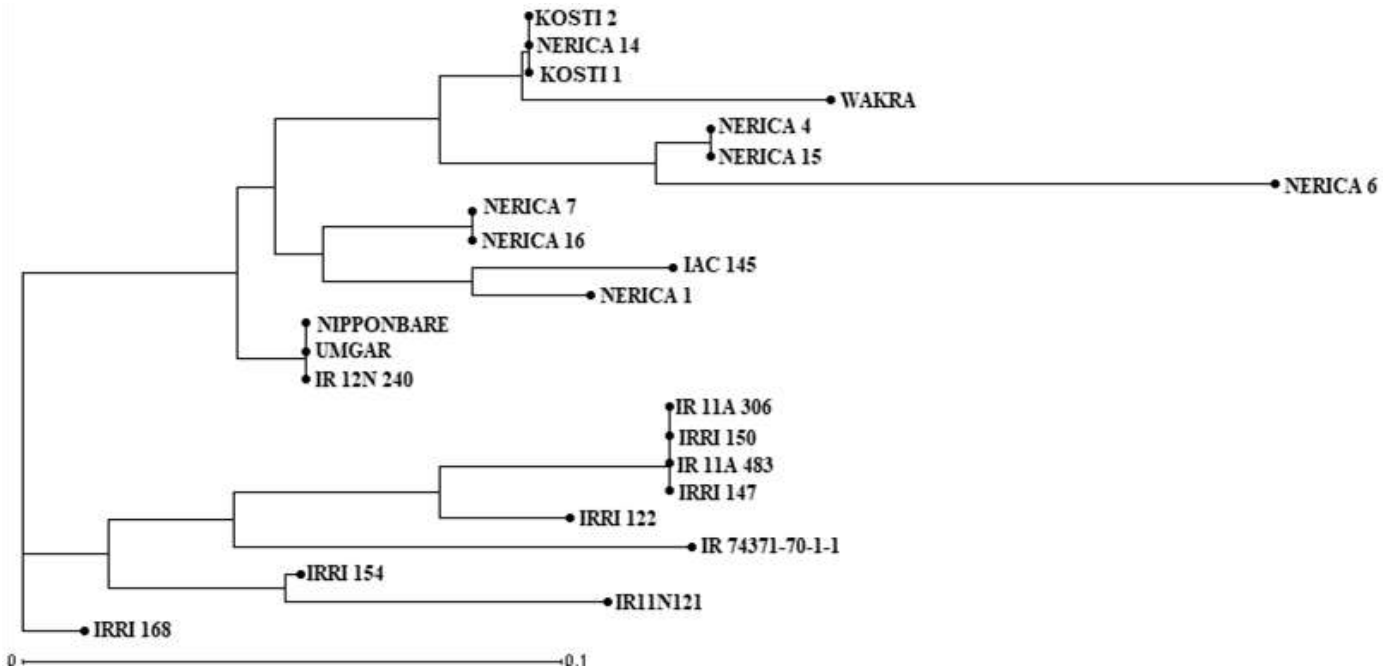

Figure 6. A dendrogram generated from UPGMA cluster analysis based on Jaccard's dissimilarity coefficient with 1000 boostrap replicates showing 23 rice genotypes generated from 18 SSR markers scoring.

chlorophyll content at the end of the dehydration period while the other genotypes showed a significant reduction. Genotypes in cluster 2 and cluster 3 are lowland. Here, IR11A306, IRRI 154 and IRRI 150 showed an increase in total chlorophyll content at the end of the dehydration period with the rest of the genotypes in both clusters showing a reduction in chlorophyll.

DISCUSSION

Effect of drought stress on total chlorophyll content

Drought stress affects morphological and physiological traits during plant growth and development. Particularly, drought stress leads to a reduction in leaf area, cell size and intercellular volume (Ndjiondjop et al., 2010). Furthermore, it has been demonstrated that exposure of plants to drought leads to a significant effect in chlorophyll contents as a result of the reduction in leaf growth (Chutia and Borah, 2012). In the current study, each rice genotype was examined under drought stress by monitoring the change in total chlorophyll content following seven days of withholding water and a further seven days of re-watering. The study data showed significant increase in levels of chlorophyll in some genotypes while others recorded marked chlorophyll decline as a result of dehydration. This could be attributed to drought's effect on chlorophyll biosynthesis and therefore a strong indication that photosynthesis would subsequently be affected in these plants. It has been previously reported that chlorophyll supports more efficient energy conversion into ATP and NADPH which are then used as source of energy to build carbohydrates from CO_2 (Pena et al., 1986). Chlorophyll degradation in these genotypes might be an indication that photosynthesis had already been inhibited when the color of leaves started to change. Consequently, some of the genotypes including IR74371-70-1-1, IRRI 168, IRRI 147, Nipponbare, Kosti 2, IAC 165 and Umgar showed recovery of chlorophyll after rehydration while IR11A306, NERICA 15, IRRI 150, IRRI 122, IR12N 240, IRRI 154, NERICA 16, NERICA 4 and Wakra showed no recovery following drought (based on the negative percentage loss of chlorophyll). Overall, these observations indicated variations among these genotypes with respect to chlorophyll levels and how the individual lines respond to drought stress. It is important for a tolerant plant to maintain high level of chlorophyll under drought stress to ensure continued photosynthesis. In cereals, higher total chlorophyll content under stress conditions has been reported and this is an indicator for drought tolerance (Gummuluru et al., 1989).

Patterns of SSR polymorphism and their correlation with chlorophyll under drought stress

The uses of molecular markers to select germplasm possessing genes and genomic regions that control

target traits can fast-track the progress of breeding for drought tolerant rice. This is because molecular markers are transmitted faithfully from generation to generation and are not subject to environmental influences (Afiukwa et al., 2016). The SSR markers are efficient along with the system of choice for genetic analysis in rice because of their abundance in the rice genome, high level of polymorphism, reliable and high but simple reproducible assays (Singh et al., 2010). For these reasons therefore, SSR markers have been used in molecular characterization of rice as well as other crop species (Semagn et al., 2006). In the current study, a total of 569 alleles were amplified using 18 SSR primers ranging between 13 and 113 alleles per primer. The study data is comparatively higher than that of Aficukwa et al. (2016) who reported between 4 and 25 alleles per primer and PIC values of 0.76 to 0.95. This reflects the high discriminatory ability of the used markers and therefore affirms their use in genetic characterization studies (Singh et al., 2010).

Improvement of rice for drought tolerance using conventional breeding methods is slow due to the differences in geographical locations and variations of seasons in drought timing and severity, the drought tolerance complex nature itself and the selection of combinations difficulty of traits (Courtois et al., 2003). Furthermore, other factors that underline the slow progress include low heritability, multiple gene control, genotype and environment interactions. All these were shown to substantially influence crop yields (Cattivelli et al., 2008). Tracking the population structure of rice genotypes under this study grouped them into 3 major clusters. The dendrogram analysis provided an evidence of the ecosystem of each genotype and the genetic relation between them as cluster 1 (with two sub-clusters) comprised 93% upland genotypes and the rain fed lowland genotypes grouped in cluster 2 and 3.

A comparison between the pattern in which the SSR markers clustered the genotypes and change in chlorophyll allowed determination of marker-trait associations for drought tolerance showed that none of the markers clearly grouped the genotypes according to the change pattern of the total chlorophyll content. A majority of cluster 1 genotypes were from the lowland ecosystem, and showed an increase in the chlorophyll content at the end of dehydration as expected result while the others showed a significant reduction. In spite of both cluster 2 and 3 being rain fed lowland genotypes, some of them showed an increase in the chlorophyll content at the end of the dehydration period. It could, therefore be deduced from the results that the markers were able to group the genotypes based on their ecosystem reflecting the strength of the SSR markers in analyzing and explaining the population genetic structure as earlier demonstrated by Garris et al. (2005). Notable, also, was the observation that although NERICA 1, NERICA 14, NERICA 15, NERICA 16 and NERICA 7 have been described as drought tolerant (Somado et al., 2008); they did not show the expected performance under the current study conditions.

Conclusion

This study noted variations among the genotypes screened with respect to chlorophyll levels and how the individual lines respond to drought stress. For determination of the levels of genetic diversity in the population, a total of 18 SSR markers were successfully amplified resulting in a total of 569 alleles with 13 to 113 alleles per primer with an average of 31.7. The PIC values ranged from 0.51 to 0.99 with a mean value of 0.88. Furthermore, comparing the pattern in which the SSR markers grouped the genotypes and the change in total chlorophyll content in each genotype, showed that none of the markers clearly grouped the genotypes according to the patterns of change in total chlorophyll contents. These results could play a role in developing genotypes that tolerate drought stress through analysis of molecular and morphological genetic diversity information thereby selecting the best parental lines for developing and improving drought tolerant rice varieties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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