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Research Application Summary

Influence of Auxin (2.4-D,NAA and TDZ) on callus induction from different explants of *Hibiscus sabdariffa*

Remaz, T., Mohammed, M. K., Sayed, F. A. A. & Sayeda, O. E.

Department of Botany and Agricultural Biotechnology, Faculty of Agriculture, University of Khartoum, Shambat, P. O. Box 32, 13314, Khartoum, North Sudan
Corresponding author: Remazbio@gmail.com

Abstract

The production of *Hibiscus sabdariffa* (Roselle) in Sudan has increased during the last few years mainly by increasing the area under production. The crop is sold in both domestic and foreign markets. It is also important as a natural coloring material for drugs and many other products. In addition, it is used as traditional medicine while oil from its seed is used as a lubricant is for lighting, making soap, paints, and varnishes. Seeds are also used for animal feeding. The crop is still produced by traditional methods under rainfed conditions and is thus affected by the uncertainty and fluctuation of rainfall. The high cost involved in implementing modern production techniques discourages adoption of improved practices. Due to these conditions, Roselle yields in Sudan are far below the potential of the crop. In this study we investigated the ability of different explants of Roselle and hormone type and concentration to induce callus to develop an efficient and reproducible regeneration protocol. Results showed that callus can be induced from *Hibiscus sabdariffa.* The best Clorox concentration for seed sterilization was 40%, the best Murashige & Skoog's medium (MS) concentrations suitable for seeds germination is full MS medium. The highest weight of callus obtained was from cotyledon explants grown in full MS media supplemented with 4mg/l of naphthalene acetic acid (NAA) hormone.

Key words: *Hibiscus sabdariffa*, regeneration protocol, roselle, Sudan, tissue culture technique

Résumé

La production d'*Hibiscus sabdariffa* (Roselle) au Soudan a augmenté ces dernières années principalement en augmentant la superficie en production. La récolte est vendue sur les marchés nationaux et étrangers. Il est également important en tant que matière colorante naturelle pour les médicaments et de nombreux autres produits. De plus, il est utilisé comme médecine traditionnelle tandis que l'huile de sa graine est utilisée comme lubrifiant pour l'éclairage, la fabrication de savon, de peintures et de vernis. Les graines sont également utilisées pour l'alimentation des animaux. La culture est toujours produite par des méthodes traditionnelles dans des conditions pluviales et est donc affectée par l'incertitude et la fluctuation des précipitations. Le coût élevé de la mise en œuvre de techniques de production modernes décourage l'adoption de pratiques améliorées. En raison de ces conditions, les rendements de Roselle au Soudan sont bien inférieurs au potentiel de la culture. Dans cette étude, nous avons étudié la capacité de différents explants de Roselle et de type d'hormone et de concentration à induire des cals pour développer un protocole de régénération efficace et reproductible. Les résultats ont montré que des

cals peuvent être induites par *Hibiscus sabdariffa.* La meilleure concentration de Clorox pour la stérilisation des semences était de 40%, la meilleure concentration de milieu de Murashige & Skoog (MS) convenant à la germination des graines est un milieu MS complet. Le poids le plus élevé de cals obtenu provenait d'explants de cotylédons cultivés dans un milieu MS complet complété avec 4 mg / l d'hormone d'acide naphtalène acétique (NAA).

Mots clés : *Hibiscus sabdariffa,* protocole de régénération, roselle, Soudan, technique de culture tissulaire

Introduction

Hibiscus sabdariffa belongs to the family (Malvaceae) commonly known as Roselle (English) and Oseille de Guinée (French). It is an erect annual herb cultivated for its seeds, petals and leaves. The red color of Roselle petals is essentially anthocyanins. It is an attractive source of natural food colorants (Abeda *et al*., 2014). The seeds are considered excellent feed for chickens, and the residue after oil extraction is valued as cattle feed (Abd-El-Bagi, 2001).

Pharmacognosts in Senegal recommend Roselle extract for lowering blood pressure. The heated leaves are applied to cracks in the feet. A lotion made from leaves is used on sores and wounds (Morton, 1987). Roselle is generally propagated by seeds. Recently, however, research was initiated to develop a tissue culture based technique for propagating this plant. Tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. This technique is used for micropropagation of many medicinal plants and in enhancing the natural levels of valuable secondary products. Cell growth and secondary metabolites can be manipulated by changing the media and culture conditions. Various juvenile as well as mature explants could produce callus when cultured in MS media supplemented with 2.4-D, NAA and TDZ hormones. In Roselle significant amounts of calli from variable explants were obtained. The objective of this study was to develop an efficient and reproducible regeneration protocol for Roselle. Specifically, the study aimed (i) to develop successful method for in vitro surface sterilization of Roselle seeds, (ii) to assess the ability of different explants as potential for callus induction, and (iii) to determine the best hormone type and concentration suitable for callus induction.

Materials and methods

This study was conducted in January 2015 at the Commission of Biotechnoloy and Genetic Engineering Department of Tissue culture laboratories in Khartoum, Sudan. Mature seeds of Roselle (local cultivar) used in this study were obtained from the Department of Agronomy, Faculty of Agriculture, University of Khartoum, Sudan.

Preparation of culture media. The media used was Murashige and Skoog (MS media). Distilled water was used for the preparation of culture media. After addition of all macro-and micro-nutrients, growth regulators and sucrose (3%) were supplemented, agar at 7.0 g/ l was used for gelling the medium. The media pH was adjusted to 5.8 using 1.0N NaOH or HCL prior to autoclaving. Media were dispensed into culture bottles covered with plastic caps. Different concentrations were prepared (full MS concentration, ½ MS and ¼ MS).

Preparation of explants. Sterilized seeds were transferred to glass bottles containing 25ml of half-strength MS medium. Seeds were incubated for one week at 25ºC± 2 under cool white fluorescent light and 16 h photoperiod. Two types of explants (hypocotyls and cotyledon) were excised from one week old seedlings under aseptic conditions in a lamina air flow cabinet and used as explants.

Methods of sterilization

The media were autoclaved at 121°C for 15 minutes at 15 psi, and stored at 25°C±2 in a growth chamber Roselle seeds were treated with H_2SO_4 for two minute to break the hard surface of seeds, then washed thoroughly with distilled water (5 times). Roselle seeds were then subjected to washes using of different concentrations of clorox® (0.5 % free chlorine) solution (20%, 40%, and 60%) for 15 minutes, followed by rinsing four times using sterile distilled water. Thereafter, the seeds were cultured in 25 ml of half-strength MS media. When seedings were 7 days old, cotyledon and hypocotyls pieces were cut and surface sterilized and used as explants. Subsequently, **t**he explants were incubated in a culture room at 25ºC±2°C under cool white fluorescent light (1000 Iux), continuous dark or under a photoperiod of 16 h light and 8 h darkness.

Sterile seeds described above were put in each Petri dishes containing double layer filter papers moistened with drops of water. Three replicates were prepared, and Petri dishes were incubated in dark for two days. Number of germinating seeds were counted and the percentage seeds germination were calculated.

Effect of different concentrations of Clorox on seeds sterilization. Roselle seeds were immersed in H_2SO_4 for two minutes to break down the hard surface of seeds then washed thoroughly with distilled water. Seeds were then surface sterilized by immersion in different 20% , 40% and 60 % Clorox® (0.5 %free chlorine) solution, with few drops of liquid soap with continuous shaking for 15 minutes. Seeds were then rinsed four times using sterile distilled water. Using forceps, four seeds were taken and placed on bottles containing ½ strength MS media in five replicates for each Clorox concentration. The bottles were incubated for one week. The number of clean seeds were calculated.

Effect of different media concentrations. Three different basal MS medium (Full- strength, 1/2 strength and 1/4-strength) were evaluated for their effects on in vitro germination of Roselle seeds. Twenty surface sterilized seeds were directly incubated on the germination medium in five culture bottles for 7 days (3 days in dark and 4 days in light at 25°C±2), with 4 seeds/culture bottle. Incubation conditions remained as those in 3.5 above. The effect of media strength was evaluated by seed germinability. .

Effect of hormones concentrations and explants on callus induction. Different plant hormones (NAA, 2.4-D and TDZ) concentrations (0.0 , 0.5 , 1.0 , 2.0 and 4.0 mgl-1) were used in the study. Thirty two (32) bottles were prepared for each hormone, with eight replicates of each hormone concentration and three explants in each bottle. All cultures were incubated at $25^0C \pm 2^0C$. Explants were allowed to grow for seven weeks.

Statistical analysis and data collection. The experimental data were subjected to analysis of variance procedure (ANOVA). Treatment means were separated using the Duncan's multiple range test (DMRT) (Duncan, 1955). Least significant differences (LSD) were computed at the 5% level of significance to compare the treatment means.

Results and discussion

Effect of different concentrations of Clorox on seeds sterilization. Results showed that the percentage of non_- contaminated seed was 93.8, 100 and 100 % respectively for 20, 40 and 60 % chlorox. Other studies have achieved total seed sterilization by immersion in 70% ethanol for three min followed by 20 min in 50% Clorox plus 0.2% Tween 20 and finally rinsed three times with sterile distilled water. For economic reasons, 40% Clorox was adapted in this study.

Effect of different concentrations of MS media on Roselle seed germination. Results showed that the percentage of seed germination ranged from 42.8, 33.3 and 19.4 % for full, ½ and ¼ strength MS medium. The high performance of full strength MS medium could be due to presence of optimum amounts of all the necessary components on the media. In cotton, similar response was achieved using full strength MS basal medium (Michel, 2008).

Effect of different hormone concentrations and explants on callus induction. Mean weights of calli produced from the different concentrations of growth hormones (0 - 0.5 -1 - 2 and 4 % mg/l) 2.4-D ,NAA and TDZ, respectively on explants were calculated and the results are shown in Table 1. The results in Table 1 show that the calli weights increased significantly with the increase in hormone concentration from 0 to 4, for the three hormones (2.4-D, NAA and TDZ). From this result it is also clear that NAA resulted into the highest mean callus growth followed by 2.4-D and TDZ. The effect of different concentrations of 2.4-D, NAA and TDZ on callus induction from cotyledon and hypocotyl explants is shown in Table 2.

From Table 2, it is clear that the effect of hormone concentration on callus induction and growth was inconsistent. There is generally an increase in callus weight with increase in hormone concentration, although the increase is not linear. NAA resulted in the highest callus weight followed by TDZ then 2.4D when cotyleydon tissue was used as explants. The same trend was seen when hypocotyl explants were used.

The high callus producing capacity of cotyledon in comparison to hypocotyls is probably due to the nutrient reserves in the cotyledons and anatomical similarity with a leaf. Our results are in agreement with those published by other authors who showed that cotyledons were more callogenic compared to hypocotyl explants. In these studies, hormone combinations involving 2,4 D were also unfavourable to callogenesis (Sle *et al*., 2010). From our results NAA too gave the highest callus weight followed by TDZ and 2.4D respectively

Conclusion

From this study it is concluded that clorox concentration of 40% is the best concentration for sterilizing Roselle seeds. The best seed germination was obtained in full strength MS media. We concluded that callus from Roselle can be induced from both cotyledonous and hypocotyl tissues, although cotyledons respond much better especially in full strength MS media supplemented with 4mg/l of NAA.

Table 1. Effect of different concentrations of growth hormones (2-4.D, NAA, TDZ) on callus induction from cotyledon and hypocotyl tissue of roselle seed

Hormone concentration	Calli weight (gm)			
		2, 4-D	NAA	TDZ
0	0.10g	0.10g	0.10g	
0.5	0.77cde	1.02c	0.50ef	
1	0.66def	1.48b	0.67def	
2	0.67def	1.63b	0.66def	
4	0.93cd	2.30a	0.43f	
	Mean	0.62	1.30	0.47

Means with similar letters are not significantly different at $p<0.05$

Table 2. Effect of different concentrations of growth hormones 2-4.D , NAA and TDZ on callus induction from cotyledon and hypocotyl explants

Hormone concentration	Cotyledon			Hypocotyls		
	2-4.D	NAA	TDZ	2-4.D	NAA	TDZ
0	0.10k	0.10k	0.10k	0.10k	0.10k	0.10k
0.5	0.20jk	0.94defg	0.36ijk	1.35cd	1.10de	0.63fghi
1	0.11k	1.15de	0.34ijk	1.20 de	1.81 b	1.01 def
2	0.26ijk	1.17de	0.50hijk	1.07 de	2.08 b	0.82efgh
4	0.15jk	2.50a	0.31ijk	1.71bc	2.10ab	0.54ghij
Mean	2.63				0.98	

Means with similar letters are not significantly different at $p<0.05$

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