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Research Article

IMPROVEMENT OF REGENERATION FROM THE ROOT PIECES OF *ALBIZIALEBBECK* BENTH. USING DIFFERENT SUGARS

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ABSTRACT

The present study was conducted to investigate effect of type and concentration of different sugars as carbohydrates sources on callus induction, direct and indirect organogenesis using roots explants of *Albizialebbeck* Benth. Sterile seeds of *A. lebbeck* were germinated on MS free medium. Roots explants were taken from 10-12 days old seedlings and transferred to MS medium contained 4.0 $g.l^{-1}$ charcoal with sugars: glucose, maltose and sucrose individually at :0.0, 20, 30 and 40 ($g.l^{-1}$). Data were recorded according to the callus induction percentage, numbers and lengths of the regenerated roots and shoots. Results showed highest percentage of callus induction (100%) was obtained at 30$g.l^{-1}$ glucose, 20 and 30 $g.l^{-1}$ maltose ; 20, 30 and 40 $g.l^{-1}$ of sucrose, whereas glucose at the concentration 20$g.l^{-1}$ gave highest mean of root number (9.64 roots), shoot number (7.0 shoots) and number of leaves (5.71 leaves/shoot), while the highest means of root length (11.9 and 11.7 mm) were recorded in sucrose at 20 and 40 $g.l^{-1}$ respectively, and longest shoot length (19.9 mm) was recorded in 30 $g.l^{-1}$ of glucose. Induced callus was sub-cultured on MS medium without charcoal and contained TDZ (1.0, 2.0 and 3.0 $mg.l^{-1}$) for regeneration. Results showed the highest fresh and dry weights (4540 279 mg respectively), and highest number of regenerated shoots (17.8 shoots) were obtained at 3.0 $g.l^{-1}$ of TDZ.

INTRODUCTION

Albizialebbeck plants are large deciduous trees, and are largely distributed worldwide, belonging to the family leaguminosae; and have valuable importance (Shirisha *et al.*, 2013). The roots of these plants form symbiotic relationships with bacteria for nitrogen fixation in the soil (Mamun *et al.*, 2004), and have a medicinal importance; i.e.: as a source of various medicinally compounds (Chaudhary *et al.*, 2012). The *in vitro* organogenesis has been employed to rapid multiplication of plants, and very important for plants improving through the genetic engineering techniques (Kothari *et al.*, 2010; Sawaensak *et al.*, 2011).

Growth of the *in vitro* cultured plant cells, tissues or organs is largely depended on the composition of culture media (organic and inorganic components and water). Sugars are the most organic components added to culture media as an important carbon source and they enter metabolic pathways for supplying energy required for cells growth and development during *in vitro* culture stages , because of the plant tissues are insufficient to achieve photosynthesis (Rolland *et al.*, 2002 ; Al-Khateeb, 2008). Generally, sucrose; is main carbon's source and energy in the *in vitro* studies for callus growth and for shoot regeneration, because it considered a common sugar in the phloem's sap of different plants (Fuents *et al.*, 2000). On the other hand, various types of sugars with different concentrations were used in different culture systems such as dextrose, glucose, mannose, sucrose, galactose, fructose, mannitol , maltose and sorbitol (Azar and Kazemiani, 2011 ; Gauchan, 2012 ; Thwe *et al.*, 2013 ; Panathula *et al.*, 2014).

The root explants have proven to be easy in manipulation, the highly regenerative rates in different species, perfect susceptible system for transformation, and in the production of active metabolites (Zobayed and Saxena, 2003), also for conservation of genetic material (Chaturvedi *et al.*, 2004). The regeneration ability of organogenesis using root explants; was achieved with different plants(Vila *et al.*, 2005; Simoes *et al.*, 2009; Arora *et al.*, 2010; Perveen *et al.*, 2011,Viana da Silva *et al.*, 2011).

Aims of this study were to evaluate validity of sugars like glucose, maltose and sucrose on: callus induction and organogenesis using root pieces of *Albizialebbeck* .

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MATERIALS AND METHODS

Collection of seeds, surface sterilization and germination

Mature and healthy seeds of *Albizialebbeck* were obtained from 10-15 years old trees growing in the gardens of Al-Musaib Technical College. In laboratory, and to facilitate seed germination; seed's coat was cut from the opposite side to the embryo with a sharp scalpel until the tissue of cotyledons was appeared and seeds were rinsed thoroughly under tap water, transferred to laminar air flow cabinet where they immersed in 0.1 % $HgCl_2$, for five minutes (for surface sterilization). Then, rinsed in sterile distilled water two times (3 minutes each). Sterilized seeds were transferred to the MS medium (Murashige and Skoog, 1962) for germination.

Explants, culture medium and incubation conditions

Root explants (0.8-1.5 cm long) were excised from 10-12 days old germinated sterile seedlings, and were cultured in hormone – free basal medium of MS containing various concentrations of the sugars : glucose , maltose or sucrose (20, 30, or40 $g.l^{-1}$). Activated charcoal added at concentration 4.0 $g.l^{-1}$ to all media, and pH was adjusted to 5.8 using 0.1 or 1.0 N of NaOH or HCl, before adding 7.0 $g.l^{-1}$of agar and poured in 15 cm × 4 cm glass jars and were autoclaved for 20 minutes at 121 ºC and 1bar pressure. Cultures, then, were incubated in culture room at 25 ± 1 ºC under 1000 Lux for 16 hrs. as photoperiod. Each concentration had 10 replicates with 3 root pieces in each replicate. Observations on callus induction frequency, number and length of induced shoots and number and length of induced roots were taken after 8 weeks of culture.

Effect of TDZ on regeneration from callus

The induced callus from previous experiment was transferred into MS medium supplemented with TDZ (1.0, 2.0, or 3.0 $mg.l^{-1}$), for regeneration from callus. Four replicates were used for each concentrations, and cultures were incubated at the same conditions mentioned above. Results of fresh and dry weights of callus, percentage of regeneration rate, and number of regenerated buds or shoots from callus were taken after 8 weeks of culture.

Statistical analysis

Using of (CRD) design for all experiments. Data analyzed with (ANOVA). Means were compared using: least significance difference (LSD) at $p \leq 0.05$ (GenStat, 2012).

RESULTS AND DISCUSSION

Effect of different sugars without any hormonal additions on the percentage of callus induction from roots explants

The present study showed that the calli were induced on root explants during 2 weeks in media supplemented with different concentrations of sugars without any additions of plant growth regulators. Results revealed highest percentage of callus induction (100%) that observed in the concentrations 30 $g.l^{-1}$of glucose, 20 $g.l^{-1}$of maltose, 20, 30 and 40 $g.l^{-1}$of sucrose, whereas the control (MS medium without carbohydrate source) gave the lowest percentage that was 42.85%(Table-1).The degree of callus induction intensity was different among different concentrations of sugars. The vigorous callus was formed in the concentration 30 $g.l^{-1}$ of both glucose and sucrose. Also, the most induced callus were white or white creamy in color and friable in texture (Table-1-, and Figure -1). It was known that sugars had important roles in *in vitro* culture media; it plays as an energy source and in the regulation of osmotic potential of culture media (Bhojwani and Razdan, 1996; Panathula *et al.*, 2014). In the study of Anwar *et al.*(2005), they found that the response of *Centellaasiatica* cultures was depended basically on sugar's type, that was supplied to cultural media, in which, maltose; was best for the stimulation of buds regeneration.

Table 1 Effect of different sugars on induction percentage and morphological features of callus induced from root explants of *Albizialebbeck* after 8 weeks of culture on MS medium

Sugars	Concentration ($g.l^{-1}$)	Callus induction (%)	Degree of callus intensity	Callus color	Callus texture
Control	0.0	42.85	+	White	friable
Glucose	20	83.00	++	White creamy	friable
	30	100.00	+++	White creamy	friable
	40	50.00	+	White creamy	friable
Maltose	20	100.00	++	white creamy	friable
	30	100.00	++	White creamy	friable
	40	85.00	+	White	friable
Sucrose	20	100.00	++	White creamy	friable
	30	100.00	+++	White	friable
	40	100.00	++	White	friable
LSD≤0.05	-	**30.53**	-	-	-

–No callus induction, + poor callus, ++ medium callus, +++ vigorous callus.

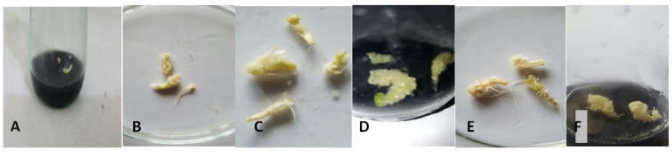

Figure 1 Different responses of root explants for callus induction with different degrees of intensity in MS medium supplemented with different sugars: A)Root explants cultured on medium containing 4.0$mg.l^{-1}$ charcoal. B)Degree of callus intensity in glucose. C) and D) Degree of callus intensity in maltose. E)and F)Degree of callus intensity in sucrose.

Effect of the type and concentration of different sugars without any hormonal additions on the root regeneration from roots explants

The results in Table-2-, and Figure-2-, showed the effect of different sugars on root regeneration from root explants. The medium that contained 20 $g.l^{-1}$of glucose; was the best for root regeneration. This concentration gave the highest mean of root number was 9.64 roots/ root explant, which differ significantly from other treatments except from the concentration 20 $g.l^{-1}$ of maltose and sucrose that were gave 7.29 and 7.66 roots/ root explant respectively, whereas, the control and concentration 30 $g.l^{-1}$of sucrose gave lowest mean of roots number (0.71 roots/ root explant for both).

On the other hand, the longest root lengths (11.9 and 11.7 mm) were obtained at the concentrations 20 and 40 $g.l^{-1}$of sucrose respectively, that overcame significantly on the other concentrations of sugars, whereas the lowest length of roots was 1.7 mm was obtained in the control treatment. The capacity of root formation was poor in medium without carbohydrates source displays the importance of sugars as an energy source, that plays a main role during tissue development of *in vitro* organogenesis and plant regeneration. This means

that the sugars do not induce directly the formation of roots from root explants, but they also stimulate the growth and development of the root primordia that were present previously in root explants (Hartmann *et al.*, 1990). So, this is the reason; why the control treatment gave the lowest rate of roots in the absence of energy source (i.e. sugars).

Roots number increased with lower concentrations of sugars (glucose, maltose and sucrose), whereas, there was a gradual reduction in this mean with the increasing of sugars concentrations. This may explain the concept of antagonism relationship between callus induction and organogenesis from explants (L'Helgoualch, 1987). Also, the sucrose was noticed to be the best for roots length among the tested sugars. The osmotic concentration of the carbohydrates source has non-positive relationship with the concentration of carbohydrates source. This relationship causes a primary elevation, followed by the decline in the means of measured characters. The decline caused by: highest osmotic level, or the carbohydrates toxicity (Thwe *et al.*, 2013).

Table 2 Effect of different sugars on means of numbers and lengths of roots that induced from root explants of *Albizialebbeck* after 8 weeks of culture on MS medium

Sugars	Concentration ($g.l^{-1}$)	Degree of roots formation	No. of roots/ root explant	Length of roots (mm)
Control	0.0	+	0.71	1.7
Glucose	20	+++	9.64	6.0
	30	+	0.86	3.7
	40	++	1.43	2.6
Maltose	20	+++	7.29	7.4
	30	++	1.00	5.7
	40	++	1.43	2.3
Sucrose	20	+++	7.66	11.9
	30	+	0.71	4.3
	40	++	4.50	11.7
L.S.D. ≤ 0.05	-	-	3.509	2.880

−No roots formation, + poor roots formation, ++ medium roots formation, +++ more roots formation.

Figure 2 Roots induction with callus on root explants in different concentrations of sugars: A) Poor root formation in control treatment (MS medium without any sugar). B) Glucose at 20 $g.l^{-1}$. C) and D)Maltose at 20 $g.l^{-1}$. E)Sucrose at 20 $g.l^{-1}$.

Effect oftype and concentration of different sugars without any hormonal additions on shoot regeneration from roots explants

The different concentrations of tested sugars affected the shoots regeneration from roots explants as shown in Table -3-, and Figure -3-. By comparing all treatments, the media containing 20 and 30 $g.l^{-1}$ of glucose, were the most effective in shoot regeneration that gave the highest means of shoots number reached to 7.0 and 6.14 shoots or buds per root explant, followed by maltose at concentration 20 $g.l^{-1}$, which gave 4.57 shoots or buds per root explant, whereas the treatments of control and 40 $g.l^{-1}$ of glucose, did not respond to regenerate any buds or shoots.

Maximum mean of shoots length (19.90 mm) was recorded in medium containing glucose at concentration 30 $g.l^{-1}$, which significantly overcame other sugars treatments, followed by the concentration 20 $g.l^{-1}$ of glucose, that gave 16.6 mm. On the other hand, the highest mean of leaves number per shoot was 5.71 leaves/ shoot was recorded at the concentration 20 $g.l^{-1}$ of glucose, which differ significantly from other treatments followed by the concentration 30 $g.l^{-1}$ of glucose, that gave 3.80 leaves / shoot.

Table 3 Effect of different sugars on means of numbers and lengths of regenerated shoots and mean number of leaves /shoot that induced from root explants of *Albizialebbeck* after 8 weeks of culture on MS medium

Sugars	Concentration ($g.l^{-1}$)	Degree of shoots or buds formation	Shoots No. / root	Shoots length (mm)	Leaves No./ shoots
Control	0.0	−	0.00	0.00	0.00
Glucose	20	+++	7.00	16.60	5.71
	30	+++	6.14	19.90	3.80
	40	−	0.00	0.00	0.00
Maltose	20	++	4.57	14.70	2.00
	30	++	1.29	0.90	0.19
	40	+	0.43	0.60	0.10
Sucrose	20	+	0.57	11.10	2.11
	30	+	0.86	15.40	2.29
	40	+	0.29	1.60	0.30
L.S.D. ≤ 0.05	-	-	2.216	3.280	1.102

−No shoots formation, + poor shoots formation , ++ medium shoots formation, +++ more shoots formation.

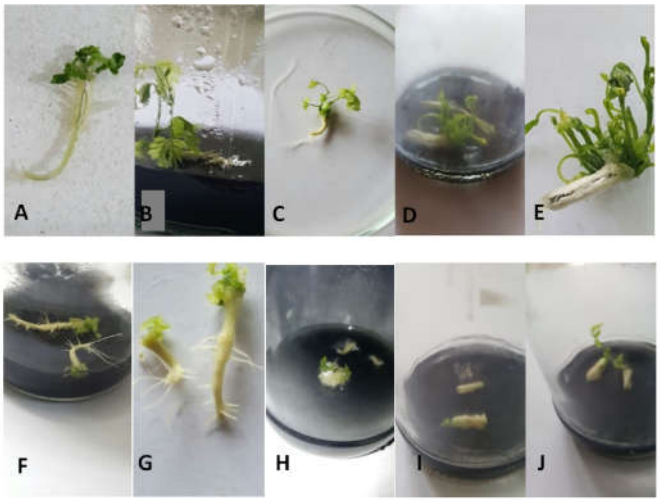

Figure 3 Shoots regeneration at different stages from roots explants cultured on medium contained sugars: A)Regeneration of shoots with roots after 4 weeks on 20 $g.l^{-1}$ glucose. B) and C)Regeneration of shoots with roots after 8 weeks on 20 $g.l^{-1}$ glucose. D) and E)Regeneration of shoots after 8 weeks on 30 $g.l^{-1}$ glucose. F) and G)Regeneration of shoots and roots after 8 weeks on 20 $g.l^{-1}$ maltose. H)Regeneration of shoots after 8 weeks on maltose (30 $g.l^{-1}$). I) and J)Regeneration of shoots on sucrose(30 $g.l^{-1}$) after 4 and 8 weeks respectively

In vitro regeneration, multiplication and growth of shoots were influenced by various factors, including: type and concentration of supplying of carbohydrates source into culture medium(Lipavska and Konradova, 2004). In previous studies, they compared the effect of many carbon sources on *in vitro* development of plants, and clear that the sucrose was more effective and appropriate for regeneration of plants of different plant species (Touqeer *et al.*, 2007; Swamy *et al.*, 2010 ; Sujana and Naidu, 2011 ; Panathula *et al.*, 2014), whereas

Anwar *et al.* (2005), reported that the addition of maltose (instead of sucrose) was the better carbohydrates source in the shoots regeneration of *Centellaasiatica*. Moreover, in the study of Sridhar and Naidu (2011) on *Solanumnigrum*, they found that from the different sugars that used in their study, the concentration 4% of fructose, was the best for numerous shoots regeneration followed by sucrose, maltose and glucose respectively. Also, Gauchan (2012) recorded that the other sources of carbohydrates such as sorbitol and mannitol, play very important role in growth of corn cultures *in vitro*. In the study of Iwase *et al.*(2011), they revealed that; the wounding method of explants is one of the factors affecting the direct organogenesis , since it induced the activity of endogenous cytokinin in explants that cultured in growth regulators-free medium.

In the present study, the glucose was the best carbohydrates source in the regeneration of maximum shoots number and length in *Albizialebbeck* root explants. The influence of glucose on the direct formation of shoots has been studied in *Prunusmume* (Hisashi and Yasuhiro, 1996). The glucose used to regulate the osmotic potential during cell division and regeneration processes. This illustrated the cleavage of sucrose during autoclaving into glucose and fructose and the utilization of glucose firstly, followed by fructose (Pierik, 1997; Murkute *et al.*, 2002). The decreasing in shoot lengths at the higher concentrations of sugars may be due to the inhibition of organogenesis that due to an excessive osmosis or carbohydrates' toxicity (Slesak *et al.*, 2004 ; Sridhar and Naidu, 2011). Thus, the important goal is to determine the optimum concentration of carbohydrates source when improving the plant regeneration techniques.

Effect of TDZ on fresh and dry weights and regeneration of plantlets from induced callus

After the transferred of induced callus into new media supplemented with TDZ for more growth of callus and regeneration from it. It was found that the addition of TDZ to the medium caused increase in the fresh and dry weights of callus (Table-4), in which; the best means of the fresh and dry weights of callus were 4540 and 279 mg respectively, that were obtained at the concentration 3.0 $mg.l^{-1}$ of TDZ ; which overcame on other TDZ concentrations . This may be due to the accumulation of sugars to form starch in callus tissues under the effect of cytokinein activity; to be used for the next processes of organogenesis (Sharma *et al.*, 1993). On the other hand, the higher percentage of plantlets regeneration rate (100%), and highest number of regenerated plantlets (17.8 plantlets), were obtained in the concentration 3.0 $mg.l^{-1}$of TDZ. Figure (4) shows the different stages of plantlets regeneration from callus at concentrations of TDZ.

Table 4 Effect of TDZ on fresh, dry weights and plantlets regeneration from induced callus of *Albizialebbeck* after 8 weeks of culture

TDZ ($mg.l^{-1}$)	Fresh weight of callus (mg)	Dry weight of callus (mg)	Regeneration rate (%)	No. of regenerated plantlets from callus
1.0	2091	131	86	6.5
2.0	2209	101	75	2.2
3.0	4540	279	100	17.8
L.S.D. ≤ 0.05	1787.7	74.1	31.04	10.37

The regeneration of plants can be achieved indirectly from unorganized callus tissue; that derived from different explants via dedifferentiation induced by exogenous supplying of plant growth regulators. The plant regeneration from callus is possible through the *de novo* organogenesis or somatic embryogenesis (Pierik, 1997). The thiadiazuron(TDZ), was used as a cytokinin in many studies for shoot multiplication, also, it was more effective than the other types of cytokinins in woody species (Lu,1993). Moreover, TDZ is used for the obtaining of higher rates of somatic embryogenesis than the other plant growth regulators. In sometimes, this cytokinin, alone; has been found to be a substitution for both auxin and cytokinin in the different plant species (Von, 2007).

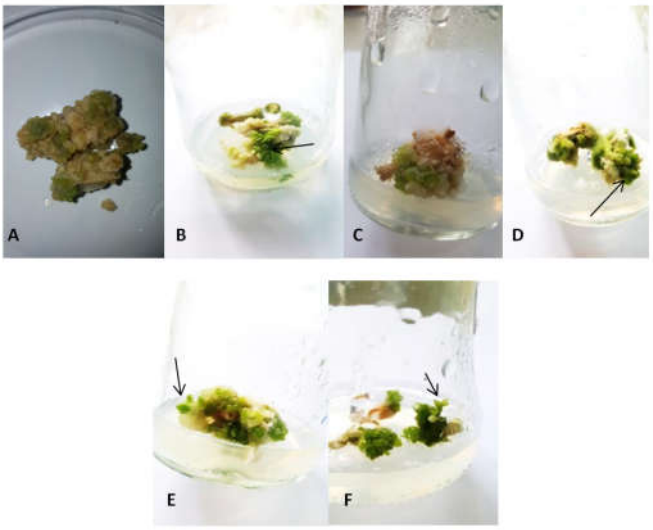

Figure 4 Stages of plantlets regeneration after 8 weeks of culture on MS medium supplemented with different concentrations of TDZ: A) and B) at 1.0 $mg.l^{-1}$ TDZ. C) and D) 2.0 $mg.l^{-1}$. E) and F) at 3.0 $mg.l^{-1}$

CONCLUSION

Results of this study, display that micro propagation of *Albizialebbeck* plants which can be achieved in short period of time through direct induction of multiple shoots from root explants excised from *in vitro* growing seedlings using various concentrations of different sugars without any addition of plant growth regulators and without any noticeable phenotypic-changes. This may support the applications of plant biotechnologies in agriculture, genetic engineering and germplasm conservation, and also can reduce the cost due to reduce in *in vitro* propagation steps and without any addition of growth regulators. Furthermore, the results introduced a successful protocol for the callus induction from roots explants, and indirect regeneration of plantlets from callus can be used for farther studies of genetic engineering and production of plant secondary metabolites.

Declaration

Authors declare that they have no conflict of interest.

Authors contributions

SihamA. Salim concepted, designed, achieved experiments , analyzed results and wrote research. Kawthr H.Abood performed experiments and recorded data. May A.Razzooqee

performed experiments and took photos of research. Authors read and appreciated final status of research.

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