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Effect of Silver Nitrate and Amino Acids on High Frequency Plants Regeneration in Barley (*Hordeum vulgare* L.)

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Key words: Barley, Immature embryos, Somatic embryogenesis

Abstract

Three Bangladeshi barley genotypes *viz.* BARI barley-1, 3 and 6 were selected for this study to evaluate the efficiency of callus induction and plant regeneration. The effect of five doses of $AgNO_3$ singly, and combined with amino acids (L-proline, L-glutamine) on callus induction and plant regeneration efficiency was evaluated using BARI barley-3 and 6. The maximum values of callus induction were recorded at 49.20 and 32.66% for BARI barley-6 and 3**,** respectively when 2.0 mg/l $AgNO_3$ and 200 mg/l L-glutamine were added to the callus induction medium. Moreover, plant regeneration remarkably increased on MS + 1.0 mg/l BAP + 1.5 mg/l $AgNO_3$ + 150 mg/l L-glutamine as 37.20% in BARI barley-6 and 16.13% in BARI barley-3. For rooting $AgNO_3$ singly affect positively, whereas negative influence was observed in combinations with any amino acids. However, by using $AgNO_3$ and amino acids, around < 4, < 27 and < 5 fold increase in callus induction were obtained. Regeneration and rooting were also found to increase considerably.

Introduction

Barley (*Hordeum vulgare* L.) is an ancient, widely distributed cereal crop in the world and this crop also grows in Bangladesh. It is used for malt, feed, food and many commercially industrial foods manufacture. A reliable improvement of barley genotypes is needed to develop through *in vitro* culture that advantageous over traditional methods of propagation. It provides a source of standardized plant material for the analysis of plant metabolism and other cellular processes

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and responses (Taji et al. 1992). Orton (1979) reported that growth and development of barley in tissue culture, depends on media used in each phase, i.e. callus initiation, callus maintenance, and differentiation of callus into shoots and roots.

The efficiency of callus formation and plant regeneration depends on the donor plant material i.e. species or cultivars (Bregitzer 1992). Moreover, callus quality varied considerably among genotypes (Vasil and Vasil 1987, Islam 2010); and most barley varieties initiate friable and translucent callus (Hanzel et al. 1985, Ward and Jordan 2001, Chauhan and Kothari 2004). Bregitzer et al. (1998) also reported that the poor regeneration potential of modern cultivars is one current limitation of barley transformation. Besides, it is an essential approach in biotechnology to improve plant genotypes. Indian barley cultivars are often considered as less responsive to tissue culture due to poor callus induction, low frequency of embryogenesis and lesser percentage of plant regeneration (Chauhan and Kothari 2004). However, regeneration ability is strongly affected by several factors such as genotypes, developmental stages and composition of culture medium and type of explants in barley (Gubišová et al. 2012, Haque and Islam 2014) and rice (Siddique et al. 2014). Different explants are used to induce callus and subsequent regeneration, such as immature embryos (Chang et al. 2003), immature inflorescence (Havrlentova et al. 2001, mature embryo (Abumhadi et al. 2005, He and Jia 2008), coleoptile (Sahrawat and Chand 2004) and seedling parts (Sharma et al. 2004). Klčová et al. (2004) reported that donor plant quality and environmental conditions are also influenced biotechnological research. However, callus induction and plant regeneration potential is highly dependent upon the genotype, culture media and growth regulators in barley and other cereal crops (Goldstein and Kronstad 1986, Bhaskaran and Smith 1990, Islam et al. 2001, Chauhan and Kothari 2004, Gürel et al. 2009, Khatun et al. 2010). The medium containing BAP in combination with 2, 4-D showed the positive effect on regeneration in barley anther culture (Cho et al. 1998).

Recently, some reports have been mentioned that use of silver nitrate ($AgNO_3$) and/ or amino acids in media, plays an important role to improve callus induction, shoot and root formation in various plants; such as wheat (Wu et al. 2006, Bouiamrine et al. 2012), maize (El-Itriby et al. 2003), sorghum (Pola et al. 2009), pearl millet (Oldach et al. 2001) and Naga chilli (Sharma et al. 2008, Bora et al. 2014). Some authors also reported that regeneration can be improved by $AgNO_3$ in both dicots and monocots (Kumar et al. 2009, Kabir et al. 2013). Hussein et al. (2004) examined that $AgNO_3$ promotes callus formation and regeneration in barley. So far as it is known that there is not enough work has been done on Bangladeshi barley cultivars using biotechnological approach.

Hence, this study has been conducted to provide a suitable and efficient protocol for callus induction and regeneration using $AgNO_3$ and amino acids through immature embryos of barley in Bangladesh.

Materials and Methods

Mature seeds of three barley genotypes *viz.,* BARI barley-1, 3, and 6 were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh and grown in the experimental field of the Institute of Biological Sciences, University of Rajshahi, Bangladesh. The seeds of milky phase, containing immature embryos were collected and used as explants.

Spikes, containing seeds of milky phase, approximately 14 - 16 days after anthesis were sterilized for 45 sec using 70% ethanol in a laminar air flow cabinet. To induce callus the embryos were aseptically dissected from the seeds, and inoculated on callus induction medium CIM containing MS supplemented with different concentrations of 2, 4-D (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l). The inoculated Petri dishes were sealed and cultures were incubated in the dark chamber at 25 ± 2°C for callus induction.

When the age of the calli was around three weeks, they were transferred to the regeneration medium (RM = MS + 1.0 mg/l BAP) and cultured under low light conditions at 25 ± 2°C together with 14/10 hrs (light/dark) photoperiods. The regenerated shoots length around 2 - 3 cm were transferred to root formation medium (RFM) that was GM (modified from MS, Islam 2000) + 1.0 mg/l IAA for better roots. The well developed rooted plantlets were transferred to pots, and acclimatized plants were transferred to field's growing up to maturity for seed collection.

To observe the effect of $AgNO_3$ and amino acids (AA) on callus induction (CI), plant regeneration (PR) and root formation (RF), five different concentrations of $AgNO_3$ singly and combinations with L-proline and/or L-glutamine were added to related media as shown in Table 1 (CIM) and Table 2 (RM and RFM). The combinations were added to CIM for callusing, RM for regeneration and RFM for rooting. The media without $AgNO_3$ and amino acids considered as control. Sucrose (30%), gelling agent agar (7%) were added to medium and pH adjusted 5.8.

The average or mean values were computed from five replicates with standard error (SE) and each experiment was repeated thrice. ANOVA and post hoc DMRT were done using SPSS 16.0 software.

Results and Discussion

Five different concentrations of 2, 4-D were examined in three studied genotypes, and the results showed that BARI barley-6 performed with highest frequency of callus induction (13.86%) in MS with 2.0 mg/l 2,4-D; followed by BRRI barley-3 (11.73%) (Fig. 2). On the other hand the lowest value was recorded for the genotype of BARI barley-1. No callusing was found in control (without 2,4-D) for studied genotypes. The effect of 2,4-D levels on the genotypes showed significant difference at $p < 0.05$.

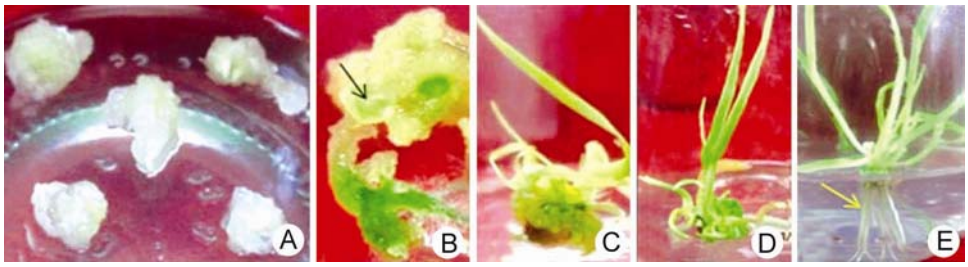

Fig. 1. Callus induction and plant regeneration of immature embryos of *Hordeum vulgare*. (A) callus induction after three weeks in CIM, (B) embryogenic callus and green spots, (C) developments of shoots, (D) well developed plantlet, (E) regenerated plants with good root and shoots.

Castillo et al. (1998) successfully regenerated plants at earlier through *in vitro* system in barley. Zapata et al. (2004) optimized 2,4-D levels as 2.0 mg/l and reported approximately similar results on callus induction using mature embryos of barley. The same level of 2,4-D (2.0 mg/l) gave the best response in maize (Jakubeková et al. 2011), wheat (Pourmohammad 2013) and chickpea (Zaman et al. 2010) which were also similar to our findings. Some studies have shown that 2,4-D is an important factor for callus initiation and proliferation of primary and embryogenic callus from immature embryos in maize (Carvalho et al. 1997, Manivannan et al. 2010). However, in the present investigation, 1.46 to 13.86% callus induction was recorded. The wide range of variability might be occurred due to genotypic effect along with the different levels of 2, 4-D. It has been reported that the frequency of callus induction, friability of embryogenic calli and regeneration are influenced by genotype, culture media and genotype × culture media interaction in barley (Bregitzer et al. 1998, Manoharan and Dahleen 2002), maize (Zhu 2011, Jakubeková et al. 2011), wheat (Farshadfar 2014, Islam et al. 2001), rice (Siddique et al. 2014, Sah and Kaur 2013), citrus (Gholami et al. 2013), sorghum (Indra and Krishnaveni 2009) and pearl millet (Jha et al.

2009). However, it could be suggested that among the 2,4-D levels (1.0-3.0 mg/l) tested, middle concentrations are comparatively better than lower and higher ones for callus induction in barley.

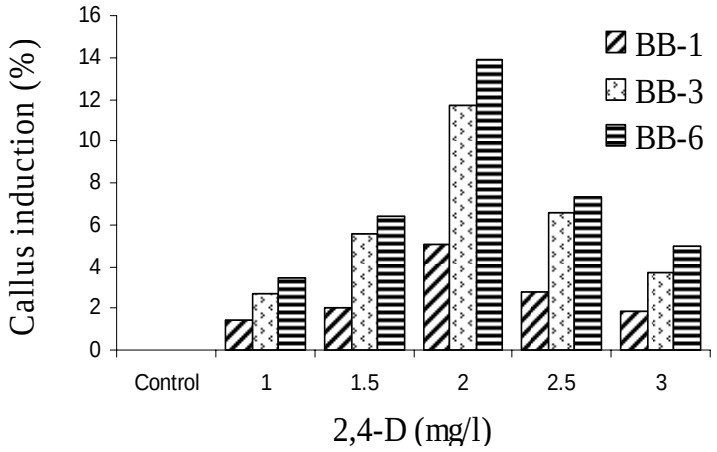

Fig. 2. Comparison of callus induction using different concentration of 2, 4-D ($p < 0.05$).

Five different concentrations of $AgNO_3$ singly and/or combined with five levels of L-proline or L-glutamine were tested, and the highest frequency of callusing was recorded in 2.0 mg/l $AgNO_3$+ 200 mg/l L-proline for BARI barley-6 (49.20%); followed by BARI barley-3 (32.66%) in the same $AgNO_3$ and amino acid concentration (Table 1). The lowest value was recorded in BARI barley-3 (9.86%) when the explants were cultured on 2.5 mg/l $AgNO_3$ + 125 mg/l L-proline + 125 mg/l L-glutamine. Whereas, the frequencies of callus induction were 11.20 and 13.24% for BARI barley-3 and BARI barley-6 in control medium, respectively. The recorded highest values were around four-folds higher than the control of BARI barley-6 and three-folds for BARI barley-3.

Fernandez et al. (1999) recorded enhanced frequency from immature embryo culture in durum wheat at large scale using $AgNO_3$. Chauhan and Kothari (2004) reported that Indian barley cultivars are often considered as less responsive to tissue culture due to poor callus induction, low frequency of embryogenesis and lesser percentage of plant regeneration. In our investigation, Bangladeshi barley cultivars also showed poor callusing in control conditions which can be overcome by addition of $AgNO_3$, L-proline and L-glutamine to callus induction medium at recommended above levels. Furthermore, different levels and combinations showed significantly different effects to studied barley cultivars. In previous report, it was suggested that concentrations of $AgNO_3$ is strongly

depended on species and genotypes (Cristea et al. 2012, Al-Khayri and Al-Bahrany 2004).

L-glutamine concentration had a noticeable effect in *Eragrostis tef* (Gugsa and Kumlehn 2011). However, most of the authors reported similar positive effect of $AgNO_3$, proline and glutamine in various plants species; such as glutamine (Hunter 1987, Mordhorst and Lörz 1993), L-glutamine, casein hydrolysate and L-proline (Ganeshan et al. 2006), casein hydrolysate and L-proline (Chauhan et al. 2007), glutamine, L-asparagine and CH (Bi and Wang 2008), aspartic acid, glutamine, proline, tryptophan and casein hydrolysate (Yu et al. 2008) proline, glutamine and asparagine (Islam and Tuteja 2012, Haque and Islam 2014). On the contrary, an inhibitory effect of glutamine has been observed in the production of barley pollen callus by Xu and Sunderland (1981).

Different combinations of silver nitrate, L-proline and L-glutamine as mentioned earlier were tested, and maximum 37.20% plant regeneration was found in 1.5 mg/l $AgNO_3$ and 150 mg/l L-glutamine for BARI barley-6. The variety BARI barley-3 performed with the frequency of 16.12% in the same level of silver nitrate and amino acids (Table 2). However, it was found that presence of silver nitrate or/and amino acids affected plant regeneration and enhanced the performance of genotypes significantly. Purnhauser et al. (1987) first reported that $AgNO_3$ (10.0 mg/l) effectively promoted regeneration in wheat. In barley cultivar Morex, almost doubled regeneration was recorded using $AgNO_3$, while 1.5-folds for Golden Promise (Jha et al. 2007). Moreover, addition of $AgNO_3$ increased the frequency of plant regeneration significantly in various plants species such as Pearl millet (Oldach et al. 2001), Naga chilli (Bora et al. 2014, Sharma et al. 2008), wheat (Bouiamrine et al. 2012, Wu et al. 2006), maize (El-Itriby et al. 2003, Huang et al. 2004), sorghum (Pola et al. 2009). In our investigation around 27-folds higher regeneration was recorded in 1.5 mg/l $AgNO_3$ and 150 mg/l L-glutamine for BARI barley-6. Anantasaran and Kamnoon (2008) found almost similar results from *Zinnia* cultivars by adding 2.0 mg/l $AgNO_3$. Gubišová et al. (2012) obtained improved regeneration and were observed significant differences in Slovak spring barley cultivars. On the contrary, no positive effect of $AgNO_3$ on plant regeneration was found using immature embryos culture in barley by Hussein et al. (2004). Pua et al. (1999) suggested that Ag^+ might interfere with polyamines. Our observations argues with their reports and suggested that $AgNO_3$ clearly affect the regeneration positively and addition of L-glutamine gives more better results especially at 1.5 mg/l $AgNO_3$ and 150 mg/l L-glutamine.

Among the concentrations of $AgNO_3$ individually added, maximum 13.06% regeneration was found in BARI barley-6 at 3.0 g/l level which was around ten-

fold higher than the control (1.40%). It was also observed that higher and lower concentrations than 3.0 g/l of $AgNO_3$, gave lower frequencies in both genotypes BARI barley-3 and BARI barley-6. Furthermore, addition of L-glutamine with $AgNO_3$ gave remarkable higher results in comparison with control. By using glutamine as a nontoxic nitrogen source, higher frequency of green plants was obtained in barley genotypes (Olsen 1987). Islam (2000) and Islam and Tuteja (2012) found that 0.5 g/l L-glutamine significantly promoted shoot regeneration in wheat androgenetic research. Glutamine and and/or proline promotes plant regeneration in microspore cultures of rice (Cho and Zapata 1988, Ogawa et al. 1995). In barley, addition of complex amino acid mixture, improved the rate of androgenic plants production in barley (Ouédraogo et al. 1998).

Table 1. Effects of different concentrations and combinations of $AgNO_3$ and amino acids on callus induction in immature embryos of two barley genotypes (% ± SE).

Supplements (mg/l)	NIE	BARI barley-3	BARI barley-6
Control	100	11.20 ± 0.58a	13.24 ± 0.81a
$AgNO_3$			
1.0	150	12.40 ± 0.91c	14.13 ± 0.99bc
2.0	150	13.06 ± 1.02cd	15.86 ± 1.01cd
3.0	150	15.33 ± 1.22e	19.73 ± 1.20de
4.0	150	14.26 ± 1.10de	17.06 ± 1.10cd
5.0	150	13.86 ± 0.77de	16.40 ± 1.14cd
$AgNO_3$ + L-proline			
0.5 + 50	300	15. 93 ± 1.40e	17.33 ± 0.89cd
1.0 + 100	300	16.80 ± 1.07ef	20.66 ± 1.47e
1.5 + 150	300	18.46 ± 1.10g	23.86 ± 1.83f
2.0 + 200	300	17.60 ± 0.83fg	21.06 ± 1.21ef
2.5 + 250	300	16.86 ± 0.67f	18.40 ± 1.13d
$AgNO_3$ + L-glutamine			
0.5 + 50	300	20.06 ± 1.06h	21.53 ± 3.31ef
1.0 + 100	300	21.13 ± 1.09i	36.53 ± 2.28h
1.5 + 150	300	23.20 ± 1.79j	40.06 ± 2.97i
2.0 + 200	300	32.66 ± 2.13k	49.20 ± 3.11j
2.5 + 250	300	19.13 ± 0.95gh	24.66 ± 1.64g
$AgNO_3$ + L-proline + L-glutamine			
0.5 + 25 + 25	150	14.80 ± 0.82de	17.73 ± 0.80cd
1.0 + 50 + 50	150	16.53 ± 1.18ef	20.53 ± 1.76e
1.5 + 75 + 75	150	13.46 ± 0.61d	16.26 ± 1.43cd
2.0 + 100 + 100	150	12.13 ± 1.12bc	15.06 ± 0.83c
2.5 + 125 + 125	150	9.86 ± 0.71b	12.13 ± 0.71b

NIE: Number of inoculated embryos. The values followed by different letters in a column are significantly different at $p < 0.05$ according to DMRT.

Efficient regeneration of plants from embryogenic barley callus often is limited to specific genotypes that exhibit vigorous plant regeneration (Bregitzer et al. 1995, Przetakiewicz et al. 2003). In our study it was observed that addition of $AgNO_3$ gave better performance than control ($AgNO_3$ > cont.). As such the influence of examined combinations could be recommended as ascending order i.e. ($AgNO_3$ and L-glutamine) > $AgNO_3$ > ($AgNO_3$, L-proline and L-glutamine) > (L-glutamine and L-proline) > control, for plant regeneration through *in vitro* culture in barley.

Table 2. Regeneration response of immature embryos, prior to $AgNO_3$ and amino acids of two barley genotypes (% ± SE).

Supplements	Plant regeneration		Rooting	
(mg/l)	BARI barley-3	BARI barley-6	BARI barley-3	BARI barley-6
Control	0. 80 ± 0.20a	1.40 ± 0.24a	2.46 ± 0.35cd	3.40 ± 0.35bc
$AgNO_3$				
1.0	5. 46 ± 0.71c	7.33 ± 0.47c	3.46 ± 0.38d	5.86 ± 0.757d
2.0	6.93 ± 0.90d	10.66 ± 1.01d	6.13 ± 0.64f	9.60 ± 0.95e
3.0	8.13 ± 0.85de	13.06 ± 1.49e	10.53 ± 1.10g	15.73 ± 1.69f
4.0	7.06 ± 0.80d	11.73 ± 1.14de	4.26 ± 0.61e	6.40 ± 0.65d
5.0	6.26 ± 0.68d	8.40 ± 0.71cd	2.40 ± 0.33cd	3.86 ± 0.57c
$AgNO_3$ + L-proline				
0.5 + 50	1.60 ± 0.24ab	2.66 ± 0.43ab	0.60 ± 0.12a	0.93 ± 0.19a
1.0 + 100	2.13 ± 0.42ab	2.93 ± 0.46ab	0.93 ± 0.19ab	1.53 ± 0.22ab
1.5 + 150	3.06 ± 0.53ab	4.13 ± 0.77b	1.33 ± 0.23b	2.06 ± 0.28b
2.0 + 200	2.26 ± 0.37ab	3.86 ± 0.53b	0	0
2.5 + 250	1.33 ± 0.29ab	2.13 ± 0.34ab	0	0
$AgNO_3$ + L-glutamine				
0.5 + 50	9.46 ± 1.42ef	15.26 ± 1.28f	0.73 ± 0.19a	1.33 ± 0.23a
1.0 + 100	12.06 ± 1.09g	24.93 ± 2.46g	1.06 ± 0.28ab	2.46 ± 0.47b
1.5 + 150	16.13 ± 1.34h	37.20 ± 2.19i	2.13 ± 0.50c	3.40 ± 0.35bc
2.0 + 200	10.66 ± 1.01f	28.46 ± 2.76h	0.66 ± 0.18a	1.06 ± 0.19a
2.5 + 250	8.40 ± 0.92e	12.53 ± 1.25e	0	0
$AgNO_3$ + L-proline + L-glutamine				
0.5 + 25 + 25	3.73 ± 0.54bc	5.46 ± 0.77bc	1.73 ± 0.33bc	2.93 ± 0.33b
1.0 + 50 + 50	5.86 ± 0.57cd	6.13 ± 0.99bc	0.93 ± 0.16ab	1.46 ± 0.24ab
1.5 + 75 + 75	3.33 ± 0.47b	4.13 ± 0.57b	0	0
2.0 + 100 + 100	2.26 ± 0.33ab	3.06 ± 0.33ab	0	0
2.5 + 125 + 125	1.73 ± 0.45ab	2.93 ± 0.49ab	0	0

The values followed by different letters in a column are significantly different at $p < 0.05$ according to DMRT.

Concentrations and combinations of $AgNO_3$ and amino acids as mentioned previously were also examined for root induction; and the maximum frequency

of rooting (10.53%) was obtained in 3.0 mg/l $AgNO_3$ for BARI barley-3 and 15.73% for BARI barley-6 in the same concentration of $AgNO_3$ (Table 2). No significant effect was observed in rooting compared to controls when amino acids were added with $AgNO_3$. Moreover, in some cases, especially in higher concentrations of and $AgNO_3$ and amino acids no root induction was found in both genotypes. Present findings agreed well with the reports as noticed in *Rotula aquatica* (Lour) where 2.67 mg/l of silver nitrate gave the improved frequency of rooting (Sunandakumari et al. 2004). Kumar et al. (2009) and Reddy et al. (2001) also observed that the positive effect of $AgNO_3$ for root formation in barley genotypes. They obtained higher values of rooting frequency using 9.12 mg/l of $AgNO_3$ in which elongation of roots were also increased. In present study, 2-5-folds increased rooting was recorded when $AgNO_3$ was individually added in various concentrations to rooting medium. On the contrary, the efficiency of $AgNO_3$ to influence the root induction was reduced when any amino acids were combined with $AgNO_3$. Silva et al. (2011) mentioned that although the effect of $AgNO_3$ is not well understood, it is supposed that the silver ion binds a possible ethylene receptor at the plasma membrane, thus inhibiting the binding of ethylene to this receptor and consequently triggering the specific action of the hormone.

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