Pak. J. Bot., 46(3): 939-943, 2014.

ENHANCED PLANT REGENERATION IN *LEMNA MINOR* BY AMINO ACIDS

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Abstract

In present study we investigated the effects of different L-amino acids on the plant regeneration from callus of *Lemna minor*, and established an efficient protocol. Among the 20 L-amino acids, only L-Ser and L-Gly showed significant improving effect, with the optimal concentration being 1 mM and 1.5 mM, respectively. A regeneration frequency of 46% was observed when the callus transferred to the regeneration medium with addition of 1 mM L-Ser for 11 days. After 26 days of cultivation, the frond regeneration achieved 100% and 94% for 1 mM L-Ser and 1.5 mM L-Gly treatment, respectively.

Introduction

Lemnaceae (duckweeds) are small aquatic herbs that are widely dispersed below or on the surface of water (Landolt, 1957; Iram *et al.,* 2012). There are 5 genera and at least 38 species in this monocotyledonous family (Landolt & Kandeler, 1987). These tiny plants reproduce typically by vegetative means, with the biomass doubling every 2 days under cultured, germfree conditions (Landolt & Kandeler, 1987; Vunsh *et al*., 2007). Duckweeds have been widely used as model plants in investigations in plant physiology, ecology and environmental biology (Landolt, 1986). As their protein content can reach up to 45% of the dry weight (Chang *et al*., 1977; Porat *et al*., 1979), they are considered to be suitable plants as bioreactors to manufacture recombinant proteins, and accordingly become an ideal gene-expression platform. It has been reported that up to 7% of soluble protein for a monoclonal antibody in transgenic *Lemna minor* was achieved (Stomp, 2005). Recently, duckweeds also cause great interests in bioenergy research to solve the challenge of climate and fuel issues (Zhao *et al*., 2012).

Genetic engineering of duckweeds can be used widely in both basic research and practical applications. With the improvement of callus induction and regeneration, the time-consuming and low-efficient problems in genetic transformation will be partially resolved. To date, nine published protocols were available for frond regeneration of duckweeds (Table 1), among which four procedures were developed for *L. minor*. These protocols are mainly focused on the effects of different basal mediums, carbohydrates, as well as phytohormones on callus regeneration. However, no information is available for the effects of amino acids on the regeneration of duckweed.

Previously, it was found that L-Pro and L-Asp could enhance plant regeneration in grain and sweet sorghum as well as *Miscanthus x ogiformis Honda Giganteus* (Rao *et al*., 1995; Holme *et al*., 1997). Recently it was also reported that the addition of amino acids stimulated the maturation of embryogenic callus and plant regeneration in the halophyte *Leymus chinensis* (Sun & Hong, 2010). As L-Ser was found earlier to induce and promote the senescence of both intact and half-fronds in *Spirodela polyrriza* (Zhu *et al*., 2004) and *L. minor*, it was considered that this amino acid might have effect on the regeneration of duckweeds. In this study, we investigated the effects of the 20 basic L-amino acids on the regeneration of *L. minor*, and finally developed an efficient and reproducible protocol with the medium supplemented with L-Ser or L-Gly.

Materials and Methods

Plant materials: A strain of *L. minor* was obtained from the lake in Xiqing District of Tianjin, and cultivated aseptically on a culture medium described by Wang and Kandeler (Wang & Kandeler, 1994). All the medium in this research were adjusted to a pH value of 6.2 with 1 M NaOH or 1 M HCl solution, gelled with 0.6% agar, and then autoclaved at 121°C for 20 min. All the experimental cultures were kept at 23±2°C under long-day conditions (16 h light and 8 h dark periods) with a light intensity of about 45 μmol m^{-2} s^{-1}.

Callus induction: 10 to 15 days old fully expanded fronds were selected as explant for callus induction. Induction medium was the B5 medium (Gamborg *et al.,* 1968) containing 1.5% sucrose, 15 mg/L dicamba (Li *et al*., 2004; Przetakiewicz & Orczyk, 2003), 3.5 mg/L 2, 4-D and 1 mg/L 6-BA.

Callus subculture: After 2-3 weeks of induction, calli appeared and the mother frond became white or yellow. Then calli were transferred to the maintaining medium for subculture. The maintaining medium was B5 medium containing 1.5% sucrose, 10 mg/L CPA and 2 mg/L 2iP. The calli were transferred to fresh medium every 2 weeks.

Frond regeneration: Calli (4-7 mm in diameter) were transferred to regeneration medium (B5 medium containing 1.5% sucrose). Regenerated fronds were transferred to liquid medium for their propagation.

Table 1. Summary of experimental protocols for callus regeneration for *Lemnaceae*.

S. No.	Species	Regeneration		Reference
		Basal medium	Regulators	
1.	*Lemna gibba*	MS; sucrose	2,4-D; 2ip	Chang & Chiu, 1978
2.	*L. gibba*	SH	BA	Moon & Stomp, 1997
3.	*L. gibba*	B5	TDZ	Li *et al*., 2004
4.	*L. minor*	FNO	2ip	Frick, 1991
5.	*L. minor*	MS; sucrose	Kin, IAA	Stefaniak *et al*., 2002
6.	*L. minor*	SH	BA	Stomp, 2005
7.	*L. minor*	B5; sucrose	Kin, IAA	Chhabra *et al*., 2011
8.	*Landoltia punctata*	WP; sucrose; sorbitol	2ip	Li *et al*., 2004
9.	*Spirodela oligorhiza*	WP; sucrose	TDZ	Li *et al*., 2004

Amino acid treatment: One kind of the 20 basic L-amino acids (1 mM) was added to the regeneration medium to study the effect of amino acid on the regeneration of plants. The amino acid was dissolved by water, filter sterilized and added to the autoclaved medium, then the medium was adjusted to pH 5.8 under aseptic conditions to avoid pH change caused by the addition of amino acid.

Different concentrations of L-Ser or L-Gly were added to the medium to determine the optimal concentration for regeneration.

Measurement of amino acid in callus during regeneration: When the callus was transferred to regeneration medium with 1 mM L-Ser and L-Gly, respectively, for 3, 6, and 9 days, samples from control and treated plant tissues were frozen in liquid nitrogen immediately after harvesting (Sepehr *et al.,* 2012). The content of L-Ser and L-Gly in the callus was determined by high-performance liquid chromatography (HPLC) as described by Zhu *et al*., (2004).

Statistics: Experiments for callus induction and plant regeneration with 60 to100 fronds on each medium were repeated at least 4 times. Callus induction frequency (CIF) and plant regeneration frequency (PRF) were calculated according to Sompornpailin & Chutipaijit (2012):

$$\text{CIF (\%)} = \frac{\text{Number of successful plant regeneration}}{\text{Number of fronds cultured for induction}} \times 100$$

$$\text{CIF (\%)} = \frac{\text{Number of successful callus induction}}{\text{Number of callus cultured for regeneration}} \times 100$$

Standard deviations of four or more experiments were checked visually by error bars, and they were shown in all graphs. Data were calculated by analysis of variance using the SAS/STAT Software (SAS Institute, 1993).

Results

Callus induction, subculture and regeneration: The selected fronds as explants were cultured on callus induction medium. After 2-3 weeks callus was successfully induced (Fig. 1a), and the CIF was over 94% (Table 2). Callus was maintained on the maintaining medium (Fig. 1b) and transferred to fresh medium every 2 weeks. When the leaf-like structure were regenerated from callus on the regeneration medium (Fig. 1c, d), they were transferred to liquid medium and then they developed normally (Fig. 1e).

Effects of L-amino acids on the regeneration: The regeneration efficiency was studied on the regeneration medium with addition of 1 mM different L-amino acid for 21 days (Fig. 2). Amino acids supplement showed significant effects on *L. minor*. Among these 20 amino acids, L-Ser ($p<0.01$), L-Gly ($p<0.05$) and L-Cys ($p<0.05$) significantly promoted regeneration, with the PRF of 93%, 78% and 68%, respectively. However, L-Ala (7%), L-Glu (12%), L-Pro (13%), L-Phe (15%) and L-Leu (16%) significantly inhibited the regeneration of callus at $p<0.01$, and Met (26%), Asn (29%) and Gln (33%) significantly inhibited the regeneration of callus at $p<0.05$. No significant effects for other amino acids were observed.

Promotion of the regeneration by L-Gly and L-Ser at different concentrations: In order to further investigate the effects of L-Gly and L-Ser, different concentrations of these two amino acids were added into the regeneration medium. When callus was cultured in the regeneration medium with the addition of 1 mM L-Ser for 11 days, the PRF achieved 46%, and this fast regeneration has never been reported in former duckweed regeneration studies. The most suitable concentration for serine was 1 mM, and the PRF was 46%, 87% and 100%, respectively, on the 11^{th}, 14^{th} and 26^{th} day (Fig. 3A). As for L-Gly, the efficient regeneration was found at the concentrations of 1.0 mM and 1.5 mM, with the PRF of 33% and 32% on the 11^{th}, and 88% and 93% on the 26^{th} day, respectively (Fig. 3B).

Changes in L-Ser and L-Gly level during regeneration: When calli were cultured on regeneration medium with 1 mM L-Ser, the endogenous L-Ser content increased with prolonging the treatment time, and it was almost 2-fold of the control content on the 6^{th} day (Fig. 4A). In this treatment, the tendency of change in endogenous L-Gly content was similar to that of L-Ser, indicating that some L-Ser had been converted to L-Gly (Fig. 4B). In contrast, under the treatment of L-Gly, the endogenous L-Gly level raised only during the early days of treatment and the increase was much less when compared to the case of L-Ser (Fig. 4B). It was surprising that the endogenous content of L-Ser increased dramatically within 3 days of L-Gly treatment and declined then afterwards (Fig. 4A). This suggested that most of L-Gly absorbed by calli were converted to L-Ser during photorespiration, which led to the promotion of frond regeneration from the callus.

Table 2. Callus induction for *L. minor*.

	Number of explants cultured for induction	Number of successful callus induction	CIF (%)
Experiment 1	651	612	94
Experiment 2	599	587	98
Experiment 3	811	771	95
Experiment 4	500	485	97

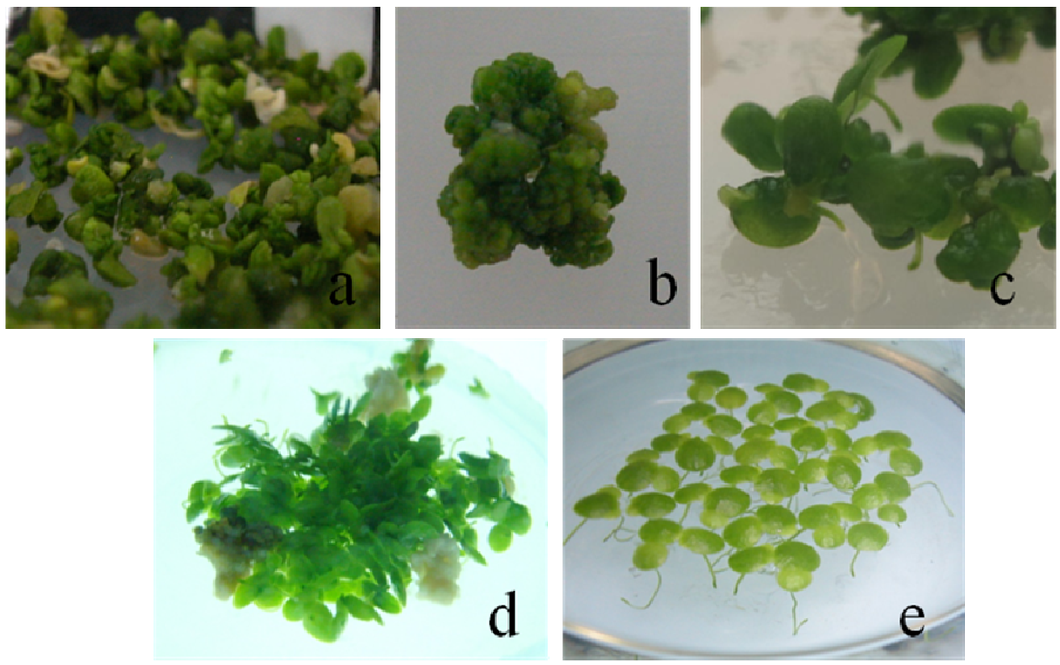

Fig. 1. Callus induction and plant regeneration of *L. minor*. **a:** callus formed on the induction medium, **b:** callus subculture on the maintaining medium, **c:** fronds regenerated from callus on the regeneration medium with 1 mM L-Ser on the 14th day, **d:** callus regenerated on the regeneration medium with 1 mM L-Ser on the 26th day. **e:** fronds propagated in liquid medium.

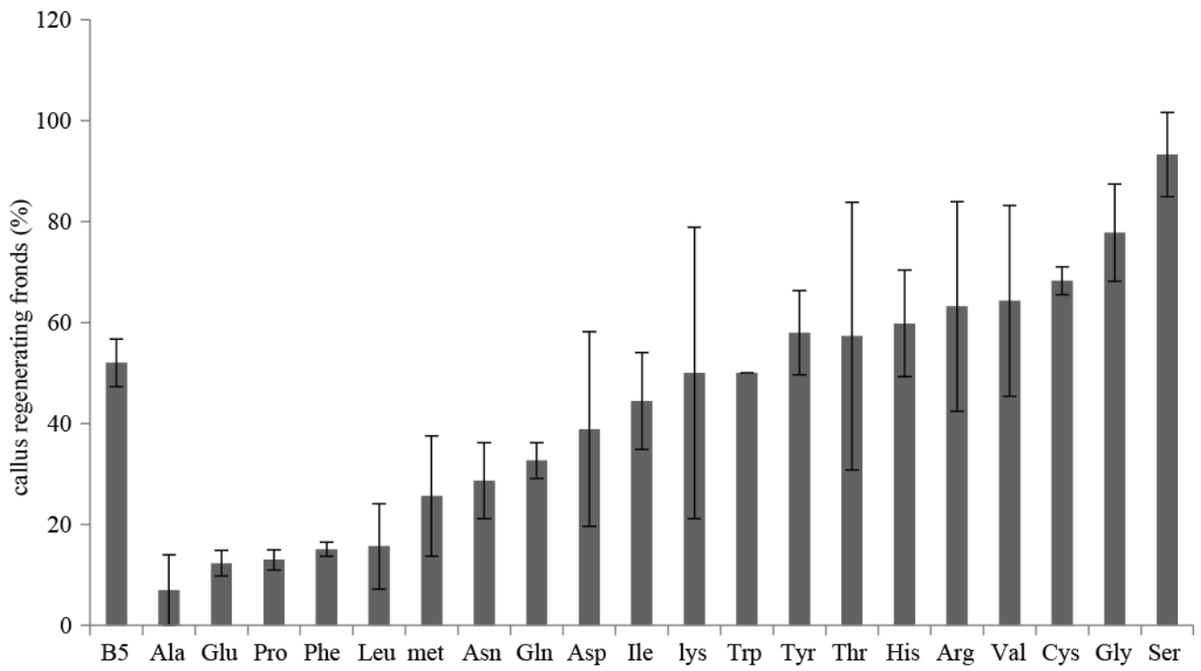

Fig. 2. Effects of different amino acids on plant regeneration from callus. Callus was cultured on regeneration medium with one kind of amino acid (1 mM) for 21 days. B5: The control, callus was cultured on regeneration medium without supplementation of any amino acids (B5 basal salt media with 1.5% sucrose).

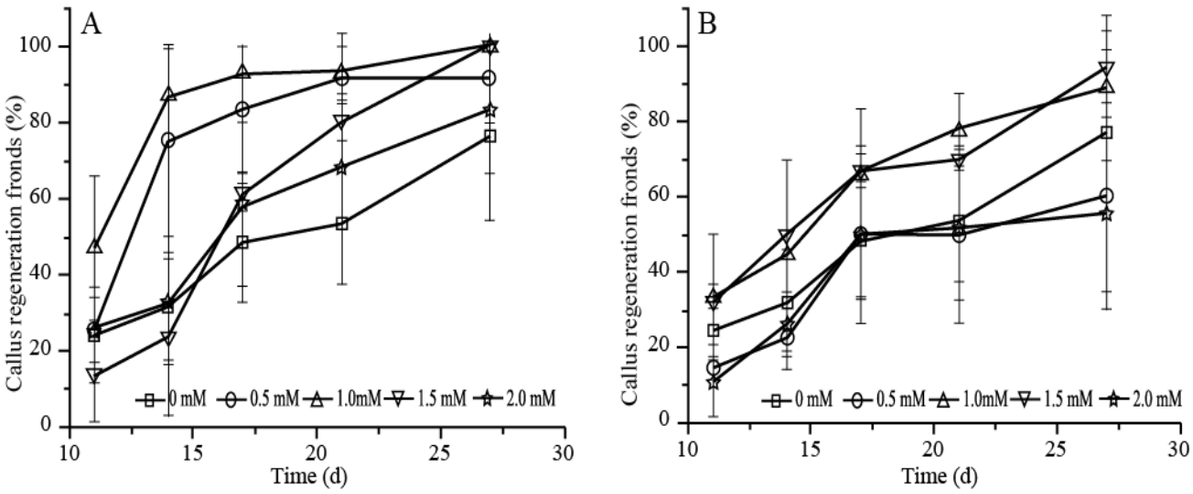

Fig. 3. Time course and frequency of regeneration from callus in the presence of different concentrations (0, 0.5, 1, 1.5, 2 mM) of L-Ser (A) or L-Gly (B).

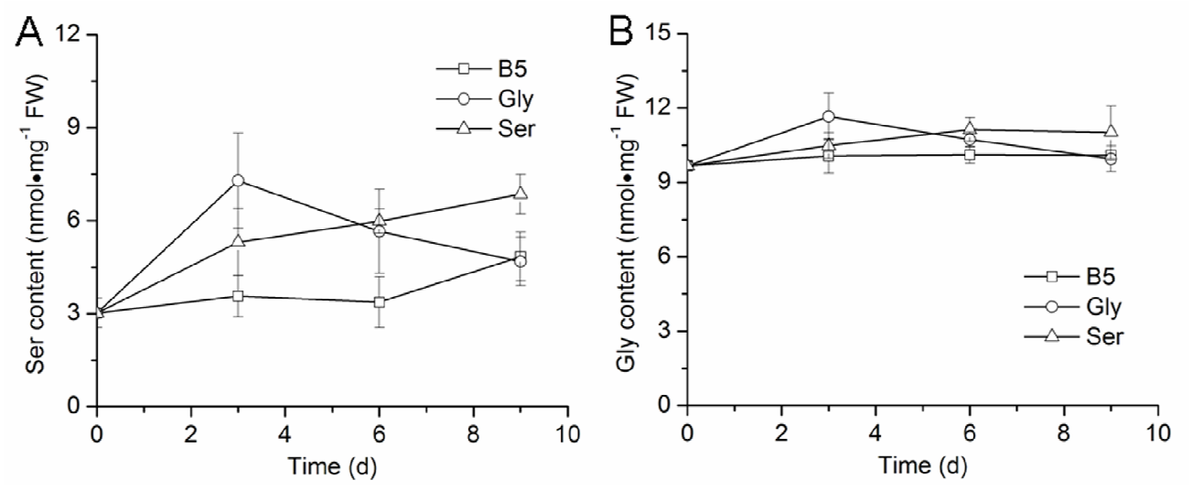

Fig. 4. Changes in endogenous L-Ser and L-Gly contents. Callus was cultivated on a medium without or with 1 mM L-Ser or 1 mM L-Gly for regeneration. A. The contents of L-Ser. B. The contents of L-Gly.

Discussion

In this study, we found that the plant regeneration from callus in *L. minor* was affected by the supplementation of the regeneration medium with some L-amino acids. Interestingly, both L-Gly and L-Ser can markedly promote the regeneration. With the addition of 1 mM L-Ser, the regeneration frequency reached 46% after 11 days of cultivation, the quickest achievement in regeneration efficiency in duckweed reported up to date. Moreover, all calli could regenerate a lot of fronds after 26 days. Although carbohydrates that poorly support the growth of the plants were reported to support the regeneration of duckweed (Li *et al*., 2004), that was approached by cultivating the callus in a 1-3% CO_2-enriched atmosphere and continuous light condition. The protocol with supplementation of L-Ser or L-Gly developed in this study should be more efficient and convenient, as the cultivation was conducted in normal atmosphere conditions.

Amino acids have been proved to be important for plant tissue culture. In grain and sweet sorghum, embryogenic callus production and regeneration frequency can be promoted by L-Asp and L-Pro (Rao *et al*., 1995). L-Glu plays a crucial role in promotion of primary callus induction, embryogenic callus formation and callus status improvement in the halophyte *L. chinensis* (Sun & Hoog, 2010), and L-Gln has been reported repeatedly to be a critical nitrogen source for embryogenesis (Wetherell & Dougall, 1976; Kamada & Harada, 1979; Ramakrishnan *et al*., 2005; Khaleda & Al-Forkan, 2006). However, exogenous amino acids may not only act as nitrogen source, because their content (1 mM) was sharply lower than the level of inorganic source (26.7 mM) in the culture medium (Gamborg *et al.,* 1968).

Among the 20 amino acids, L-Ser showed the best effect on the promotion of regeneration in *L. minor*, and L-Gly had the second-best effect (Fig. 2, Fig. 3). It is well known that Gly can be converted to Ser in the photorespiration pathway (Ho & Saito, 2001). The results of the changes in endogenous levels of L-Ser and L-Gly during regeneration of plants on the regeneration medium with one of these two amino acids showed that the

promotion effect of L-Gly on regeneration should be due to its conversion to L-Ser (Fig. 4). Therefore, L-Ser should be an important factor involved in the plant regeneration from *L. minor* callus. This conclusion is in accordance with the proposal that, at least in duckweeds, L-Ser may play signal roles, as it was found to induce the senescence process of *S. polyrrhiza* 143 (Zhu *et al*., 2004). However, the signal pathway should be experimentally elucidated. L-Ser signal pathway may correlated with ABA signal transduction pathway (Bertomeu *et al*., 2010a; Bertomeu *et al*., 2010b), as the serine deficiency mutant was ABA insensitive. In *Eudendrium racemosum* (Hydrozoa, Cnidaria), light stimulated endogenous ABA synthesis, and ABA in turn enhanced regeneration by the signal pathway, light-ABA-PKA3-cyclase activation-cADPR-Ca^{2+}-regeneration (Puce *et al*., 2004).

Acknowledgements

This research was supported by the National Natural Science Foundation of China (No. 30870185, No. 31270296) and partially by Tianjin Municipal Science and Technology Commision (No. 11ZCKFSF01200).

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(Received for publication 15 May 2012)