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Effect of Exogenous Plant Growth Regulators on Embryonic Development of *Vigna radiata* (Mung Bean): Differential Expression of Amylase in Immature and Mature Embryos Cultured *In vitro*

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Abstract: The precocious germination of excised embryos from immature and mature seeds at different developmental stages was studied taking into consideration the expression of α-amylase. Effect of growth hormones on enzyme activity was also analyzed in an attempt to outline their contribution to the process of embryo maturity and germination. Immature embryos excised from developing seeds of ages 7,10 and 12 Days After Flowering (DAF) were cultured *in vitro* in presence of different plant growth regulators viz, GA_3, ABA, IAA and 2,4-D, administered singly along with one combination i.e., of GA_3 and ABA. Amylase activity showed a strong, negative impact of ABA, GA_3 and their combination in the premature embryos of age 7 and 10 DAF. This inhibition pattern was consistent up to the near mature embryos of age 12 DAF, thereby preventing precocious germination and keeping embryos in the embryogenetic mode. The response to GA gradually changed from inhibition to induction as embryonic age advanced. Amongst the two auxins applied, IAA was less inhibitory than 2,4-D at younger ages, while in the near mature set the two auxins induced amylase significantly. Embryonic axes removed from dormant seeds and treated similarly showed response to ABA and GA comparable to that in the premature and near mature sets. Maximum activity was recorded in the GA_3 treated set while minimum amylase was observed in ABA. The auxin IAA at lower concentration induced amylase in the mature set while $IAA_{100\mu M}$ and 2,4-D caused strong inhibition. These results were significant at $p<0.05$. It is suggested that the release of amylase from the embryonic axes of developing seeds is inhibited by ABA and GA_3 until the embryos reach the near mature stage, the enzyme being induced in pre-mature and mature embryos by gibberellic acid, which may be the growth regulator released from the seed.

Key words: Embryogenesis, precocious germination, phytoharmones, hydrolytic enzymes

INTRODUCTION

In normal seed development, germination does not usually occur until the embryo has completed its entire development within the seed. If immature embryos are removed from the seed and cultured on nutrient medium, they have an adverse effect on normal development and germinate precociously[1].

The phytohormone Abscisic Acid (ABA) plays a variety of roles in plant growth and environmental response including a critical role in the development of seeds. A rise in ABA level during embryogenesis triggers processes that lead to the acquisition of dessication tolerance and seed dormancy[2]. In culture, the embryos can reversibly enter or leave the maturation loop by application or removal of ABA[3]. ABA is believed to antagonize a positive GA signal that induces precocious germination and also suppresses maturation phase gene expression[4]. Thus GA and ABA antagonism controls a decision point between precocious germination or quiescence and maturation in pre maturation embryos of barley, wheat and maize[5,6].

Before it begins to elongate, the embryonic axis appears to rely predominantly on reserves stored within the axis itself[7]. The growing axis utilizes primarily as food, the reserves stored in adjacent tissues[8]. Enzymes involved in the hydrolysis of storage molecules thus become prevalent in the seedlings.

The increase in α-amylase activity depends on gibberellins and the embryonic axis[9], is affected neither by excision of axis[10] nor by gibberellic acid[11] and the presence of embryonic axis as a requirement for normal developmental sequence[12] have been suggested.

Present research was conducted to study the stages of embryonic maturation, the effect of exogenous plant growth regulators on maturation *vs* germination response of developing embryos and the involvement of growth regulators and the embryo itself in the trigger of events that lead to germination and early growth of mung bean.

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MATERIALS AND METHODS

Plant material: Mung bean (*vigna radiata* (L) Wilczek), cv.19-19, was used as the test material. Seed stock was supplied by the Pakistan Agricultural Research Council Islamabad and replicated in the fields of Genetics department in April 2003.

To obtain embryos at different ages of maturity, open flowers were tagged in young, blooming plants of age 20-21 days and the pods harvested at ages 7, 10 and 12 Days After Flowering (DAF).

Immature embryos: Embryos were collected in cold sterile water upon separation of cotyledons and gentle removal of the axis with the help of forceps. Culture plates were prepared with 1.5 % agar containing any one of the following growth regulators viz., GA_3 (10 μM), ABA (10 μM), GA_3 10 μM + ABA 10 μM, 2, 4-D (0.001 μM) and IAA (2 μM) along with 1.5% agar plates used as control. Embryos were grown in culture for five days at an optimum of 37°C under aseptic conditions.

Mature embryos: Dry, desiccated seeds were immersed in distilled water overnight to soften the coats and embryonic axis removed as described above. Five days culturing in agar plates was done with above combinations of growth regulators and control.

Estimation of α-amylase: Amylase was estimated with minor modifications[13] .

Statistical analysis: Analyses were done with SPSS for MS Windows version 11.0 (SPSS, Inc., Chicago, IL). Analysis of variance was performed for amylase activity and growth regulator treatment considering the four different developmental stages of the embryos. Means of enzyme activity for each treatment were compared using Duncan's Multiple Range (DMRT). $p<0.05$ was considered significant.

RESULTS

Amylase activity in immature embryos: In general amylase activity is maximum in the 7 days old embryos and gradually decreases as they mature through 10 to 12 days (Table 1). The effect of phytohormones applied *in vitro* remains strongly negative during the early stages but gibberllic acid and the auxins cause promotion in the near mature set of age 12 days. In embryos of age 7 DAF, maximum amylase activity is found in the control set, while minimum value is observed for ABA, GA and their combination GA+ABA. The two auxins as mentioned have a lesser impact in reducing embryonic amylase. In embryos of age 10 days the overall effect of growth regulators is still inhibitory with strongest inhibition caused by the hormonal combination GA and ABA. Abscisic acid alone also continues to cause a strong inhibition of amylase. The influence of IAA, however, is less inhibitory than the synthetic auxin 2,4-D. It is important to note that in 10 days old embryos gibberellic acid administered singly has caused minimum but significant inhibition of amylase compared to the rest of the hormones. At the age of 12 days the pattern of response of embryos to applied growth regulators has somewhat changed and inhibition is now replaced by induction in case of GA_3 treated and auxin treated samples. Highest enzyme levels are recorded in GA_3 10 μM treated samples followed by 2,4-D 0.001 μM and IAA 10 μM. The inhibitory behavior of ABA is again prominent singly as well as in combination with its antagonist GA_3. Statistical analysis of the data is shown in Table 2 and 3, indicative of significance at $p\leq0.05$ and 0.01.

Amylase activity in mature embryos: The response of mature embryos to applied growth regulators (Table 1) shows two patterns i.e., induction and inhibition. Among the hormones that increase amylase activity are GA_3 and IAA while ABA and 2,4-D have a negative effect. Maximum induction is found for the two concentrations of GA_3 i.e., 10 μM and 100 μM and for IAA applied at a conc. of 10 μM for the mature embryos. In contrast, the higher conc. of IAA used i.e., 100 μM and the synthetic auxin 2,4-D had a negative effect. Here again, ABA caused strong inhibition in order to maintain seed dormancy. Statistical analysis of this data is shown in Table 2 and 3 which indicates significance at $p\leq0.05$ and 0.01.

Table 1: Amylase activity (μg maltose/mg protein/5 min) in embryos at different ages of maturity

Age	Control	2,4-D 0.001 μM	ABA 10 μM	GA_3 10 μM	GA_3 10 μM +ABA 10 μM	GA_3 100 μM	IAA 2 μM	IAA 10 μM	IAA 100 μM
7 DAF	1008.92± 8.77	766.21±16.21	133.82±2.97	150.15±2.15	169±1	nd	659±3.50	nd	nd
10 DAF	668.68±2.73	383.31±-3.35	307.88±0.88	626.99±1.57	287.02±1.66	nd	601.30±1.30	nd	nd
12 DAF	585.62±4.37	691.18±-1.12	245.83±2.91	711.92±1.92	333.05±1.84	nd	648.35±2.20	nd	nd
Mature	191.26±0.79	151.27±-1.27	124.93±2.73	280.54±1.47	270.30±1.07	279.96±1.28	nd	241.73±1.735	137.03±0.675

Values are mean±SE, nd: not determined

Table 2: ANOVA of amylase activity (µg maltose/mg protein/5 min) in immature and mature embryos

		MS values				
		Immature				
Source of variation	df	7 DAF	10 DAF	12 DAF	df	Mature
Between treatments	5	287626.4949**	59381.5187**	78199.3086**	7	8829.3939**
Within treatments	6	122.1698	10.4911	13.6014	8	4.93622

** Significance at $p \leq 0.01$

Table 3: DMRT on mean amylase activity (µg maltose/mg/5 min) in embryonic tissue cultured with PGRs at different stages of maturity

Age	Control	2,4-D 0.001 µM	ABA 10 µM	GA_3 10 µM	GA_3 10 µM +ABA 10 µM	GA_3 100 µM	IAA 2 µM	IAA 10 µM	IAA 100 µM
7 DAF	1008.92^{d}	766.21^{c}	133.82^{a}	150.15^{a}	169^{a}	nd	659^{b}	nd	nd
10 DAF	668.68^{f}	383.31^{c}	307.88^{b}	626.99^{e}	287.02^{a}	nd	602.60^{d}	nd	nd
12 DAF	585.62^{e}	691.18^{e}	245.83^{a}	711.92^{f}	333.05^{b}	nd	648.35^{d}	nd	nd
Mature	190.47^{d}	151.27^{c}	124.94^{a}	280.54^{g}	270.30^{f}	279.96^{g}	nd	241.73^{e}	137.03^{b}

Means in a row with different letters are significant at $p \leq 0.05$ level
Means with similar letter are non-significant, nd: not determined

DISCUSSION

Current data clearly shows that the immature, near mature and mature embryos of mung seeds are all capable of germinating precociously upon *in vitro* culturing. The growth response measured in terms of amylase activity is different according to the phytohormone supplied in the culture medium. Whereas in the developing embryos of 7 and 10 days, the impact of hormonal treatment is strongly negative, a positive response is found in the near mature embryos (12 days) and the mature embryos taken from dormant seeds. Throughout the period of embryonic development and germination the role of two hormones, GA and ABA is worth noting while the auxins, participate mainly in inducing amylase at the late stages of embryonic development.

Considering the GA profile, it can be seen that during the early stages of embryonic development its effect remains inhibitory, only to cause prominent amylase induction as the embryos enter the near maturation stage and finally move towards maturity and dormancy. Exogenous GA treatment down regulates maturation associated gene expression in culture[14]. In maize embryos at the late maturation stage exogenous GA_3 treatment caused rapid and complete germination[6].

The impact of ABA at all developmental stages is strongly negative. It can be seen that exogenous ABA added to the culture medium tends to reduce amylase activity in the pre-mature, near mature and fully mature embryos keeping them in the embryogenetic mode and preventing precocious germination. Also it is noticeable that reduction in amylase activity by ABA is stronger for younger, developing embryos compared to the fully mature ones. Isolated embryos of many species have been shown to suppress germination and either initiate or maintain expression of maturation-associated genes when they were cultured in presence of exogenous ABA[15]. When ABA is added in the culture medium at a concentration of 10 µM or higher, it prevents precocious germination of soybean embryos at all stages of development[16]. The addition of ABA to mature, non-dormant, seeds of *Arabidopsis* inhibits their germination. This effect of ABA might be related to its natural function as an endogenous inhibitor of precocious germination during seed formation[17]. Northern blots of maize embryos at the pre-maturation phase cultured for three days in medium supplemented with exogenous ABA, showed presence of ABA-inducible mRNAs, which are undetectable in the corresponding controls[4]. Thus results of the present study are consistent with the proposed roles of the two hormones in embryo and seed development. As for the combination of GA with ABA used in the culture medium, the latter largely mimics the response of ABA used singly, bringing down the amylase levels to a smaller value in the immature and near mature sets. An exception, however, is observed in the mature embryo culture where the effect is inductive with respect to enzyme activity. Pre mature maize embryos cultured for three days with 10 µM ABA and 10 µM GA_3 showed an accumulation of all the maturation phase mRNAs the 10 µM GA_3 only moderately diminishing their levels, suggesting that GA signaling is less effective in overcoming ABA signaling when ABA concentrations are high[6]. Finally, the inductive behavior of GA + ABA in promoting germination related activities in mature embryos may be explained by considering the fact that whereas the free ABA content is highest in developing seeds it is generally relatively low or even undetectable in mature seeds[18]. In the present case, therefore, a still higher concentration of exogenous ABA may be required to over come the positive GA signal for germination. It may be noted that the concentration of both GA and ABA in our case is 10 µM, respectively. A slightly higher concentration of ABA with GA could have resulted in a better GA/ABA balance. Thus the present induction of amylase in mature embryos by this combination may be explained.

The effect of exogenous auxins on embryonic development is inductive only during the late maturation phase i.e. the near mature and fully mature embryos, while at the younger stages auxins curtail amylase but to a relatively lesser extent compared to the strong inhibition caused by GA and ABA. Also the lower conc. of IAA (2 μM and 10 μM) when added to the culture medium is more successful than the higher IAA concentration of 100 μM. In angiosperms, early and mid stages of embryogenesis show high levels of IAA, with the early stages promoted by low concentration of auxin and inhibited by high concentration[3]. Recent findings from experimental studies of *Brassica* and *Arabidopsis* developmental mutants indicate a possibility that auxin may have a role in early embryogenesis[19]. A more critical examination of the embryonic response to many different concentrations of auxin may further highlight the role of the latter in mung bean embryonic development.

Finally it may be concluded that in mung bean, amylase is an active enzyme of embryonic development. The impact of plant growth regulators on enzyme induction is negative at first until the embryos have completed their development and are ready to germinate. Abscisic acid induces primary dormancy in mung bean embryos right from the very beginning and maintains its effect till maturation and post maturation phase. Gibberellic acid and auxins on the other hand enable the embryos to germinate precociously but this is effective only near the late stages of embryonic development.

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