Pak. J. Bot., 44(6): 1823-1829, 2012.

α-AMYLASE ACTIVITIES DURING SEED DEVELOPMENT AND GERMINATION IN PEA *(PISUM SATIVUM* L.) TREATED WITH SALICYLIC ACID

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Abstract

The effects of salicylic acid on α-amylase (EC 3.2.1.1) activities during seed development and germination in pea (*Pisum sativum* L.) were investigated. The crops were planted in split-split plot fashion following randomized complete block design (RCBD) with three replicates. The main plots were assigned to pea cultivars Climax, Meteor, Greenfeast and Rondo, with salicylic acid concentrations (0mM, 0.1mM and 0.01mM in water) as subplots and modes of application of salicylic acid (seed treatment; seed treatment plus foliar spray and foliar spray only) as sub-sub-plots. Samples were collected at phenological stages i.e., BBCH 73, BBCH 77, BBCH 83 and BBCH 88. The seeds obtained from the crop were used for germination experiment. The samples were collected at phenological stages BBCH 01, BBCH 03 and BBCH 05. Although a significant difference was recorded for varieties with respect to α-amylase activity during year 2003-04 and 2004-05 at all phenological growth stages. A non-significant difference was found for salicylic acid concentrations and modes of application. However a significant difference in α-amylase activity was observed for salicylic acid concentrations during both years at all phenological growth stages for BBCH 01, BBCH 03 and BBCH 05.

Introduction

Seed development is complex process comprising of a series of events involving cell division, cell differentiation and storage of macromolecules. In legume cotyledons, cell differentiation starts in certain regions and progressively spreads to other parts, thereby building up a developmental gradient. Seeds accumulate starch, storage proteins and oil in different proportions depending upon the species. These products are synthesized in the storage organs, the endosperm or the cotyledons, mainly based on imported sucrose and amino acids. Because of its economic importance, seed metabolism and especially the accumulation of storage products, became a subject of intensive investigation. The large sized legume seeds allow a combination of physiological, biochemical and molecular approaches with an analysis of the underlying developmental processes. Seed development is closely connected with seed metabolism and transport processes seed-filling phase when the storage reserves are deposited whereas the earlier stages of seed development have been given less consideration (Weber *et al*., 1998). To analyze how seed metabolism is connected with growth, development and control of biosynthetic pathways requires an integrated experimental approach. This includes biochemical and histological methods as well as transgenic approaches and genomic tools. The seeds of *Vicia faba* or *Pisum sativum* offer excellent models for a wide range of different methods (Borisjuk *et al*., 2003).

Physiological and biochemical aspects of fruit and seed development in large seeded legumes have been intensively studied, leading to comprehend the major events of seed formation and maturation (Rafique *et al.*, 2011; Eeuwens & Schwabe, 1975; McCarty, 1995; Cag *et al.,* 2009). The germination and seedling establishment are the most critical periods of the plant life. Seed development and maturation may be viewed as a preparation to withstand these periods. Thus, a massive and coordinated storage of nutrients is to occur during seed formation, upon which successful seed desiccation, dormancy and germination are dependent. Biochemical changes in relation to seed desiccation and dormancy have been extensively studied (Blackman *et al*., 1992; Eeuwens & Schwabe, 1975). The insights gained from physiology and modelling are being extended by the application of molecular techniques to identify and determine the function of genes expressed in association with germination (Welbaum *et al*., 1998).

Starch, a primary product of photosynthesis in higher plants is storage carbohydrate that supports metabolism and growth during the dark when photosynthesis is not possible (Zeeman *et al*., 2004). In some plants, about half of the photoassimilated carbon is stored as starch, to be remobilized later. The enzyme most frequently credited with the initial attack on starch granules is α-amylase (Trethewey & Smith, 2000). This enzyme is responsible for initiating the mobilization of starch in germinating seeds (Fincher, 1989).

Plant growth regulators comprise a large group of endogenous and exogenous chemical compounds that can regulate plant growth in numerous ways. Although phytohormones, like abscisic acid and gibberellins, regulate the metabolic pathways during seed development and seed germination respectively, salicylic acid may have regulatory effect on these two important phases in the life cycle of pea. Salicylic acid is widely distributed in angiosperms and has a role in defence mechanism in plants. Recent reviews have demonstrated that salicylic acid has a role in the control of several physiological and biochemical processes in plants (Raskin, 1995; Gross & Parthier, 1994). Salicylic acid regulates some aspects of disease resistance and thermogenesis (Sarwar *et al.,* 2010; Raskin, 1992). The aim of the current study was to evaluate the effects of exogenously applied salicylic acid on the activities of α-amylase during seed development and germination in pea (*Pisum sativum* L.) varieties.

Materials and Methods

Plant materials, experimental design and SA treatment: Four varieties of pea (*Pisum sativum* L.) viz., Meteor, Climax, Green feast and Rondo were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. The crops were planted in split-split plot fashion following randomized complete block design (RCBD) with three replicates. The main plots were assigned to pea cultivars, with salicylic acid concentrations (0mM, 0.1mM and 0.01mM in water) as subplots and modes of application of salicylic acid (seed treatment; seed treatment plus foliar spray and foliar spray only) as sub-sub-plots. Each sub-sub-plot measured 3 x 1.8 m^2 (5.4 m^2) with plant to plant distance 15 cm and row to row distance 90 cm (Hussain & Badshah, 2002). There were two rows in each sub-subplot and 20 plants in each row. Weeding, hoeing and other agronomic practices were carried out uniformly as and when needed. Three concentrations of aqueous SA (0mM, 0.01mM and 0.1mM) with pH 5.5 were applied. For seed treatment seeds were soaked in the solutions of 0mM, 0.01mM and 0.1mM concentrations of salicylic acid for six hours (Benavides-Mendoza *et al*., ,2002). The plants were sprayed at phenological stage BBCH 60 (first flower open sporadically within the population) with aqueous solutions of 0mM, 0.01mM and 0.1mM SA in the early morning when the plants had their 3rd leaf completely expanded. The sprays in all cases were carried out with a manual pump (Gutierrez-Coronado *et al*., 1998).

Samples were collected at three different phenological growth stages i.e., BBCH zero (germination), BBCH seven (fruit development) and BBCH eight (ripening of fruit and seed), respectively (Weber & Bleiholder, 1990; Feller *et al*., 1995). For BBCH 7 and 8, the pods were collected at BBCH 73 (30 percent of pods reached average maximum length), BBCH 77 (70% of pods reached average maximum length), BBCH 83 (30% of pods ripe, dry and hard) and BBCH 88 (80% of pods ripe, dry and hard).

The seeds obtained from the crop were used for germination experiment. The seeds were surface sterilized (70% ethanol for 1 minute and 5% Sodium hypochlorite for 5 minutes) and thoroughly rinsed in distilled water. The seeds were sown in plastic trays filled with the same soil that was used for cropping. For phenological growth stage zero, the samples were collected at BBCH 01 (beginning of seed imbibition), at BBCH 03 (seed imbibition complete) and at BBCH 05 (radicle emergence from the seed). All samples were stored at -50□ C in a deep freezer**.** The data were subjected to analysis of variance using the MSTAT-C program and Duncan's Multiple Range Test was applied to differentiate the means (Steel *et al*., 1997).

α -Amylase assay: α-Amylase activity was determined according to the method of Jones & Varner (1967). Seeds were extracted in 0.2 M citrate buffer (pH 5.5), centrifuged at 10,000 *g* and the supernatant was used for enzyme assay. Then 0.2 ml of the enzyme extract was diluted to make the volume 1.0 ml with distilled water. The reaction was started by the addition of 1.0 ml of starch substrate for one hour. The starch substrate was prepared by the addition of 150 mg potato starch in 100 ml of solution containing 600 mg KH_2PO_4 and 200 µmol $CaCl_2$. The mixture was boiled for 1 minute, centrifuged for 10 minutes at 3,000 *g* and clear supernatant was used as the substrate. The reaction was stopped by the addition of 1 ml of iodine reagent (6 g of KI and 600 mg of iodine were dissolved in 100 ml of water, before use 1.0 ml of the stock solution was added to 0.05 N HCl and made the volume to 100 ml). To this reaction mixture, 5.0 ml of distilled water was added, mixed and measured the absorption at 620 nm. The α-amylase activity was calculated as the amount of starch hydrolyzed per minute per mg of protein. Protein content was determined according to the method of Bradford (1976) with BSA as a standard.

Results and Discussion

The effects of SA on the activities of α-amylase were observed at phenological stage BBCH 73, 77, 83 and 88 during the years 2003-04 and 2004-05 (Table 1, Fig. 1). The varieties were significantly different in terms of activity of α-amylase during the years 2003-04 and 2004-05 at all four phenological growth stages (Table 1). The variety Rondo revealed highest while Climax exhibited lowest activity of α-amylase (Fig. 1a, b). SA treatment was non-significant at all four stages of seed development (Table 1, Fig. 1c, d). Modes of application were found non-significant during the year 2003, while a significant difference was recorded at BBCH 83 and 88 stages for the year 2004-05 (Table 1, Fig. 1e, f).

The activities of α-amylase of the seeds raised from the SA treated plants were assayed at three different stages of seed germination i.e., BBCH 01, BBCH 03 and BBCH 05 respectively during year 2003-04 and 2004-05 (Table 2, Fig. 2). During the year 2003-04 the variety Meteor exhibited maximum activity of α-amylase at all three phenological stages while the maximum α-amylase activity was recorded for the variety Greenfeast at all three phenological stages during the year 2004-05 (Fig. 2a, b). The maximum α-amylase activity was recorded at BBCH 01, BBCH 03 and BBCH 05 for the plants treated with SA 0.1 mM during the year 2003-04 and 2004-05 (Fig. 2c, d). The modes of application were found nonsignificant during the both years in terms of α-amylase activity at three phenological stages (Fig. 2e, f).

Starch is a primary product of photosynthesis in higher plants, which supports plant metabolism and growth in the absence of light. In legumes, up to half the photo-assimilated carbon is stored as starch. The perturbations of starch metabolism can have far-reaching consequences, reducing plant growth and have an affect on development (Caspar *et al*., 1991; Corbesier *et al*., 1998). There is lack of understanding of how starch is remobilized, and of how both synthetic and degradative pathways are controlled and integrated with other pathways of metabolism. Knowledge of the pathway of starch degradation is a prerequisite to understanding its

regulation. Starch breakdown in plant tissues is controlled in a way that integrates the release of carbohydrates for subsequent utilization, principally for sucrose synthesis and respiration (Fondy & Geiger, 1982). Many enzymes are capable of participating in starch degradation. The enzyme most frequently credited with the initial attack on starch granules is α-amylase (Beck & Ziegler, 1989; Ziegler, 1988). The present study revealed the maximum activity of α-amylase which was observed for the variety Rondo as compared with other varieties. Moreover all the varieties exhibited maximum α-amylase activity at BBCH 83 growth stage (Fig. 1). The SA did not affect the α-amylase activities at all four phenological stages of pea studied. The α-amylase activities were highest at phenological stage BBCH 77and declined towards phenological stages BBCH 83 and BBCH 88.

Table 1. Mean squares from the analyses of variance and coefficient of variation (C.V.) of activities of α-amylase (µmol starch hydrolysed min^{-1} mg^{-1} protein) in pea (*Pisum sativum* L.) varieties treated with salicylic acid concentrations by different modes of application during year 2003-04 and 2004-05 at four stages of fruit and seed development.

Source of variation	Df	BBCH 73	BBCH 77	BBCH 83	BBCH 88
			Year 2003-04		
Replications	2	0.02	0.68	0.19	0.02
Varieties	3	3.86**	5.54***	3.67***	6.80***
Error a	6	0.17	0.22	0.09	0.16
Salicylic acid	2	0.02	0.17	0.16	0.02
V x S	6	0.09	0.34	0.28	0.09
Error b	16	0.12	0.21	0.11	0.12
Mode	2	0.03	0.05	0.17	0.03
V x M	6	0.05	0.33	0.20	0.04
S x M	4	0.03	0.39	0.12	0.03
V x S x M	12	0.04	0.37	0.19	0.04
Error c	48	0.2	0.30	0.17	0.02
C.V.		8.21	23.88	9.70	6.37
			Year 2004-05		
Replications	2	0.34	0.32	2.55**	0.82
Varieties	3	12.37***	49.35***	1.14*	11.25*
Error a	6	0.13	0.12	0.17	1.57
Salicylic acid	2	0.22	0.20	3.29*	3.86*
V x S	6	0.33	0.34	0.55	0.69
Error b	16	0.13	0.15	0.58	0.87
Mode	2	0.12	0.14	13.67***	5.40**
V x M	6	0.10	0.09	0.84	0.45
S x M	4	0.18	0.17	0.41	0.28
V x S x M	12	0.25	0.24	0.87	0.32
Error c	48	0.21	0.20	0.58	0.76
C.V.		15.43	7.09	18.16	22.82

*, **, *** significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively

BBCH 73: Thirty percent of pods have reached average maximum length

BBCH 77: Seventy percent of pods have reached average maximum length

BBCH 83: Thirty percent of pods ripe, dry and hard

BBCH 88: Eighty percent of pods ripe, dry and hard

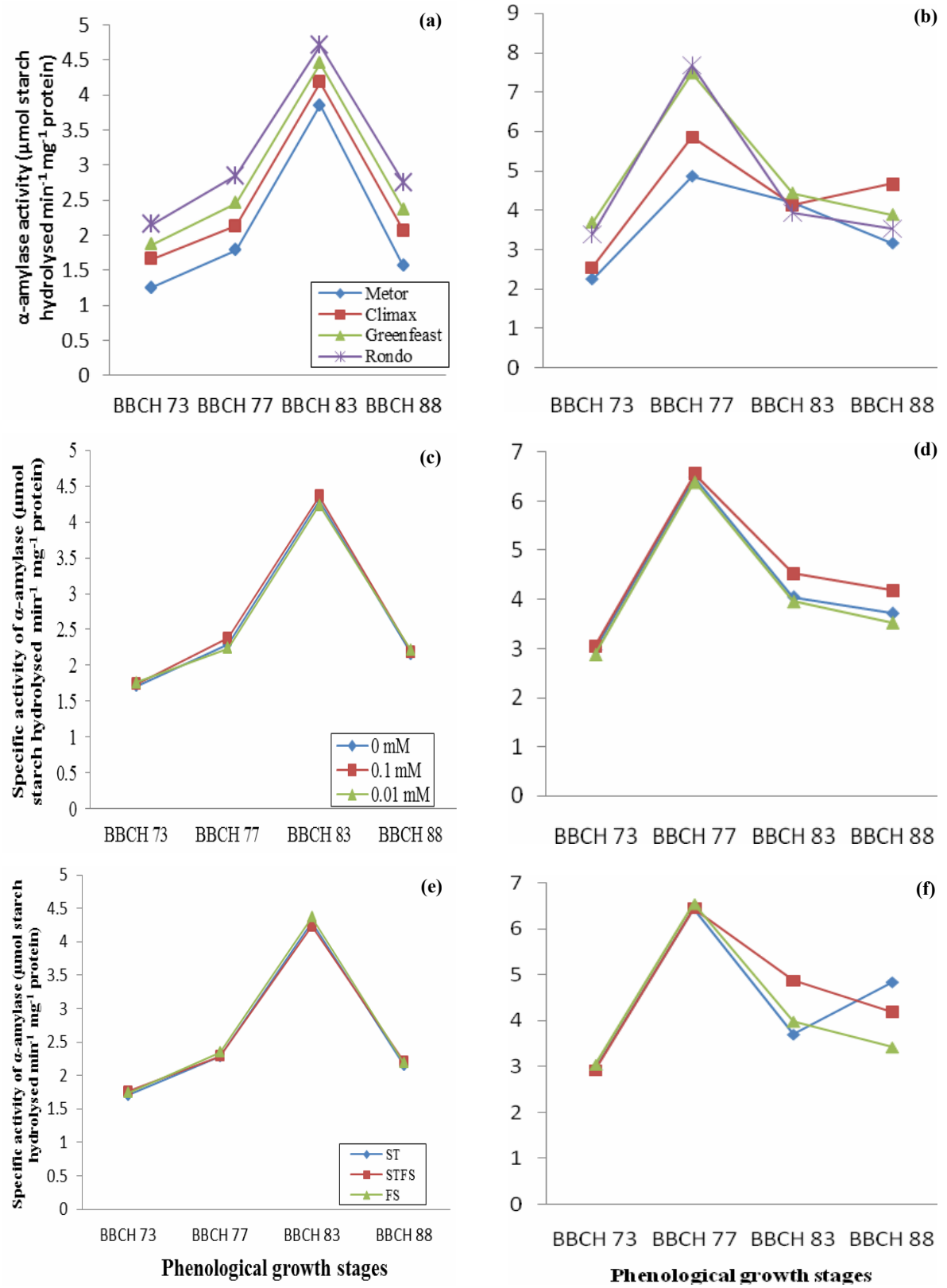

Fig. 1. Changes in the activity of *α*-amylase in four varieties (Meteor, Climax, Greenfeast and Rondo) (a, b), three salicylic acid concentrations (0, 0.1 and 0.01mM) (c, d) and of three modes of application (Seed Treatment (ST), Seed Treatment plus Foliar Spray (STFS) and Foliar Spray (FS) (e, f) in SA treated pea plants at four phenological growth stages (BBCH 73, 77, 83 and 88) during the year 2003-04 (left panel) and 2004-05 (right panel).

Table 2. Mean squares from the analyses of variance and coefficient of variation (C.V.) of α-amylase activities (µmol starch hydrolysed min^{-1} mg^{-1} protein) in pea (*Pisum sativum* L.) varieties treated with SA concentrations by different modes of application during year 2003-04 and 2004-05 at three stages of seed germination.

Source of variation	Df	BBCH 01	BBCH 03	BBCH 05
		Year 2003-04		
Replications	2	0.05	0.48	0.32
Varieties	3	5.56***	11.47***	133.07***
Error a	6	0.16	0.16	3.22
Salicylic acid	2	4.03***	4.12***	73.60***
V x S	6	0.03	0.15	5.02
Error b	16	0.10	0.21	2.03
Mode	2	0.10	0.52	0.59
V x M	6	0.08	0.06	0.23
S x M	4	0.09	0.38	0.40
V x S x M	12	0.10	0.34	0.83
Error c	48	0.06	0.29	0.46
C.V.		4.89	5.83	5.19
		Year 2004-05		
Replications	2	0.04	0.04	0.39
Varieties	3	22.32***	22.25***	133.81
Error a	6	0.17	0.15	3.25
Salicylic acid	2	4.12***	4.21***	74.20***
V x S	6	0.03	0.03	5.02
Error b	16	0.09	0.08	1.94
Mode	2	0.08	0.10	0.52
V x M	6	0.09	0.10	0.18
S x M	4	0.08	0.07	0.35
V x S x M	12	0.09	0.10*	0.84
Error c	48	0.05	0.05	0.48
C.V.		7.51	3.69	6.02

*, **, *** significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively

BBCH 01: Beginning of seed imbibition

BBCH 03: Seed imbibition complete

BBCH 05: Radicle emerged from seed

α-Amylase has an active role in the hydrolysis of the starch during seed germination. It may also be responsible for the maintenance of requisite water potential, by providing solute sugars during the seed germination phase. Strong starch degrading activity is present in the cell wall of plants. The α-amylase activity was enhanced by prolonged darkness (Saeed & Duke, 1990), leaf infection with virus (Heits *et al*., 1991), heat stress (Commuri & Duke, 1997) and water stress (Ashraf *et al*., 1995). These results suggested that the amylases were involved in the growth regulation as well as in stress resistance.

During the present study, all the four varieties were significantly different from each other in terms of α-amylase activities, which may be attributed to the difference in starch content of the varieties. The α-amylase activities were higher in the seeds raised from the pea plants treated with SA concentration 0.1 mM. The α-amylase activities during seed germination were higher as compared to during seed development (Fig. 2). The starch degradation was very slow during the phenological growth stages BBCH 01, BBCH 03 and BBCH 05; suggesting that the active mobilization of starch in pea cotyledons commences after the radicle has started to elongate. In general the pattern of starch degradation is in conformity with earlier reports on the seed germination of other plants (Uno-Okamura *et al*., 2004).

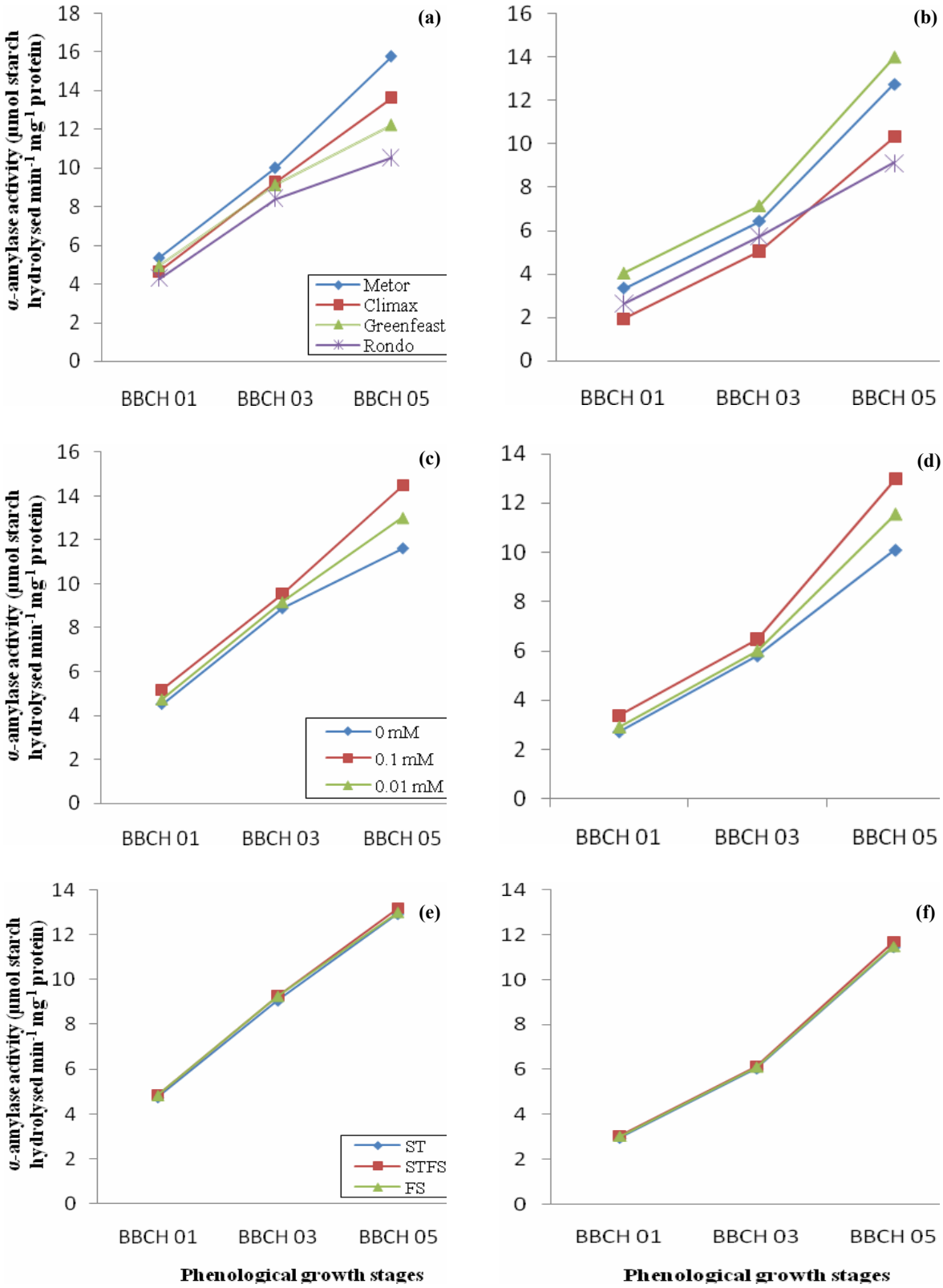

Fig. 2. Changes in the activity of α-amylase in four varieties (Meteor, Climax, Greenfeast and Rondo) (a,b), three SA concentrations (0, 0.1 and 0.01mM) (c, d) and three modes of application (Seed Treatment (ST), Seed Treatment plus Foliar Spray (STFS) and Foliar Spray (FS) (e, f) in SA treated pea plants at three phenological growth stages (BBCH 01, 03 and 05) during the year 2003-04 (left panel) and 2004-05 (right panel).

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(Received for Publication 2 August 2011)