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Ethylene inhibitors promote male gametophyte survival in rice

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Abstract

Rice (*Oryza sativa* L. cv. Lalat) was grown in pots under open field conditions during the wet season of 1997. Attempts were made to manipulate the growth and development of the male gametophyte, located on the basal region of the panicle, by exogenous application of chemicals regulating formation/action of ethylene and compare grain setting in the spikelets bearing few grain. Application of ethylene action ($AgNO_3$) and synthesis inhibitor ($Co(NO_3)_2$; paclobutrazol and uniconazole) improved grain setting in the spikelets and the ethylene releasing substance 2-chloroethyle phosphonic acid (CEPA) depressed it compared to the control. The ethylene inhibitors promoted dry mass accumulation and concentrations of starch and reducing sugars in the anthers of the basal spikelets, while CEPA reduced the level of these carbohydrates significantly. The ethylene inhibitors helped in the survival of more numbers of pollen in these anthers, but CEPA depressed their number significantly. Promotion of growth of the basal anthers was accompanied by a concomitant reduction in the concentration of nonreducing sugars and enhanced activities of acid invertase and sucrose synthase enzymes. It is concluded that male gametophyte development of the basal spikelets of rice is susceptible to ethylene at the stage of pollen mitosis. The possibility of the hormone interfering in carbohydrate metabolism of the anther during this stage of development is discussed.

1. Introduction

The heterogeneous architecture of the inflorescence of rice has been a severe impediment to maximising its grain yield potential. The panicle bears a large number of spikelets during ontogeny, but all of them do not reach maturity to produce good quality grains. The spikelets located on the apical primary branches reach anthesis first and fill properly to produce larger and heavier grains, compared to the proximal spikelets. The proximal spikelets are either sterile or fill poorly to produce grains unsuitable for human consumption [21, 26]. The metabolic dominance of the superior apical spikelets and the attendant correlative inhibition of development of the inferior proximal spikelets may be mediated through endogenous hormones. The action of such hormones may be responsible for the positional variation in development of spikelets on the panicle and should be investigated properly to enhance yield from the proximal region of the inflorescence. The review of Bangerth [1] has emphasised the role of IAA as the primary correlative signal responsible for variation in fruit/sink dominance in some plants, but the role of ethylene has been undermined. The sequence of spikelet development in rice panicles is multi-directional [20, 39], and there is a need for a highly mobile hormone for correlation of organ development. Ethylene, being a small molecular weight substance, suits for easy diffusion and fast mobility.

The role of natural hormones in correlative development remaines elusive; hardly any attempt has been made to study the mechanism in rice. There is evidence that ethrel (2-chloroethyle phosphonic acid) application induces abnormality of anther development, leading to sterility of pollen grains [2, 10]. Similarly, Morgan [22] emphasised the role of ethylene as a stress induced sporocide. This proposition was contested by Saini [32]. He stated that more ethylene is necessary for abnormal male gametophyte development than could be endogenously produced by the plant. The flag leaf of rice produces a large amount of ethylene at the time of reproductive development

[16, 31] and its impact on floral morphology of the inflorescence is unknown. As the male gametophyte is more sensitive to stresses or chemical treatments, compared to the female [15, 41], it will be of interest to manipulate growth of this organ in order to improve grain yield from the proximal region of the panicle by the application of chemicals inhibitory to ethylene action or formation.

2. Materials and methods

2.1 *Plant material and cultivation*

A semidwarf high yielding *indica* rice cultivar, Lalat (120 days), was cultivated in pots (33 × 33 × 26 cm) containing farmyard manure supplemented with N, P_2O_5 and K_2O (80: 40: 40). The fertilisers were applied in three split doses in the basal medium. The pots were kept in open field condition during the wet season of 1997. The plants were free from any biological or physico-chemical stress. A trial experiment was done initially during the dry season of 1997, but this set of data has not been presented because of similarity of responses to the chemical treatments given.

Thirty day old seedlings were transplanted into the pots for cultivation. Eight plants were grown in each pot. The six treatments were arranged with three replicate pots in a randomised block design.

2.2 *Chemical treatments*

The plants were treated with chemicals, such as $AgNO_3$ (10^{-5} M), $Co(NO_3)_2$ (10^{-5} M), 2-chloroethyl phosphonic acid (10^{-5} M), paclobutrazol (100 μL/L), (Zeneca ICI Agrochemical Ltd., Madras, India) and uniconazole (0.5 mg/L) (gift from Professor R. A. Fletcher, University of Guelph, Canada). The control plants received only distilled water. The chemicals were dissolved in distilled water and 0.5 ml of the solution was injected carefully from the top into the boot of the flag leaf sheath containing the panicle using a 1 ml syringe at the time of booting. The treatments were given early in the morning after sunrise. The chemical treatments were continued at 24 h intervals for four more days.

The first sampling was done on the day of growth regulator application. The flag leaf sheath was opened partially and anthers from the top spikelet (spikelets located on the tip of the apical three primary branches) and basal spikelet (spikelets located on the tip of the proximal secondary branch of the basal three primary branches) were removed from the panicle. On each occasion, three sets of plant material were collected from each replicate treatment. The first and second sets, consisting of all six anthers of each of the spikelets, were used for dry weight and carbohydrate estimations, respectively, both at the top and basal positions. The third set of anthers obtained from the spikelets were used for measuring enzyme activities. Very often it was necessary to obtain anthers from two basal spikelets instead of one, to get measurable enzyme activities. A fourth set of anthers was obtained from the plants at the anthesis stage for counting the number of pollen grains.

2.3 *Counting of pollen grains*

All six anthers of the spikelet were dissected. The anther wall was ruptured by application of pressure to release the pollen grains. The pollen grains were washed out of the anther wall carefully with distilled water and collected in a centrifuge tube. The sample was centrifuged at 1500 rpm for 10 min and the supernatant was discarded. The residue containing the pollen grains was resuspended in distilled water, stirred vigorously with a vortex mixer. An aliquot of the emulsion containing the pollen grains was placed in a plankton-counting chamber and counted using microscopy.

2.4 *Estimation of soluble sugars*

The anthers obtained from the spikelets were weighed fresh and immediately plunged into boiling 80% aqueous methanol in a test tube. After 5 min the extract was transferred to a volumetric flask and the residue was extracted for second time with 50% aqueous methanol. The two extracts were pooled in a volumetric flask and the final volume was made up to the mark with distilled water. An aliquot was used for analyses of sugars. The total soluble sugars in the extract were determined with anthrone reagent, according to the method of Yemm and Willis [40]. Reducing sugars were estimated according to method of Nelson [24]. The difference between the total and reducing sugars was calculated for estimation of nonreducing sugars.

2.5 *Estimation of starch*

The residue obtained after methanolic extraction was hydrolysed using 3% HCl at 100 °C for 3 h and the volume of the extract was noted. The glucose released

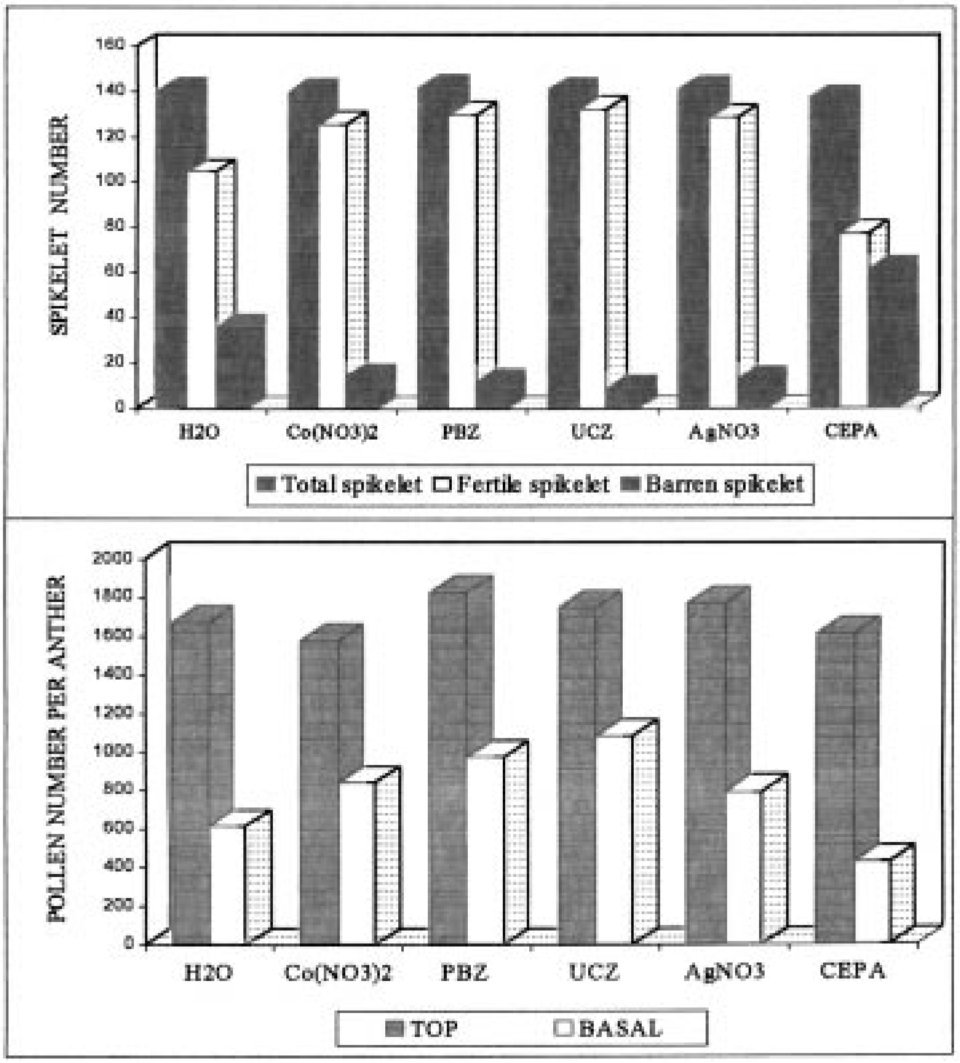

Figure 1. The effect of ethylene inhibitors (($Co(NO_3)_2$, Paclobutrazol, Uniconazole and $AgNO_3$) and an ethylene releasing substance (CEPA) on spikelet number of the panicle and pollen number of anthers (at anthesis) in rice cv. Lalat.

after acid hydrolysis was measured by the phenol sulphuric acid method of Buysee and Merck [5] for the estimation of starch.

2.6 *Estimation of sucrose synthase and acid invertase activities*

The fresh weight of anthers was recorded and the material was homogenised immediately in an ice cooled mortar with 5 ml of 50 mM Hepes buffer solution (pH 8.0) containing 8 mM $MgCl_2$, 2 mM EDTA, 50 mM 2-mercaptoethanol, 12.5% (v/v) glycerol and 5% (w/v) insoluble polyvinylpyrrolidone-40. The homogenate was centrifuged at 12000g at 4 °C for 15 min. The supernatant was collected in a 5 ml volumetric flask and the volume was made up to the mark with buffer. An aliquot of the stock solution was used for measuring sucrose synthase and acid invertase activities.

Sucrose synthase was assayed by measuring sucrose synthesis from fructose and UDPGlucose. Enzyme solution (50 μl) was incubated for 15 min at 30 °C with 2 μM fructose and 1 μM UDPGlucose in 90 μl of 50 mM Hepes-NaOH buffer (pH 7.5) containing 15 mM $MgCl_2$. The reaction was terminated by the addition of 120 μl of 1 N NaOH [36]. Unreacted fructose was destroyed by heating the mixture in a boiling water bath for 10 min. The concentration of

Table 1. t-Values indicating the influence of ethylene inhibitors and ethylene releasing substances on spikelet number of the panicle and number of pollen in the anthers at anthesis of rice CV. Lalat.

	CEPA	$Co(NO_3)_2$	PBZ	UCZ	$AgNO_3$
Total spikelets	0.753^{NS}	0.159^{NS}	0.329^{NS}	0.076^{NS}	0.060^{NS}
Fertile spikelets	4.00*	2.925 *	3.722 *	3.183*	3.556*
Barren spikelets	3.05*	2.782*	3.007*	3.448*	2.927*
Pollens at anthesis					
Top	0.620^{NS}	0.988^{NS}	1.325^{NS}	0.812^{NS}	1.084^{NS}
Basal	3.216*	3.510*	4.691*	4.497*	2.880*

Values are significantly different from control at $^{*}P < 0.05$, ^{NS} = not significant using t-test.

sucrose was determined according to the colorimetric method of Roe [30] using 1% resorcinol reagent.

For measuring acid invertase activity [34], 10 μl of enzyme solution was incubated for 10 min at 37 °C with 200 μl of reaction mixture containing 100 mM sucrose in 200 mM acetate buffer (pH 4.8). The reaction was stopped by adding 0.5 ml of Nelson copper reagent and the concentration of the reducing sugar liberated was determined according to the method of Nelson [24]. The reaction stopped at zero time served as the blank.

3. Results

3.1 *Phenology, pollen number and grain setting*

The top spikelet emerged out of the flag leaf sheath and reached anthesis in three days following the first application of the chemicals. Hence, they did not receive direct chemical treatments for the subsequent two days. Compared to the top, the basal spikelets emerged five days later and received chemical treatments for five days. Emergence coincided with anthesis in most of the treatments. In some basal spikelets, application of $AgNO_3$, $Co(NO_3)_2$, Paclobutrazol and uniconazole shortened the time of anthesis (extrusion of anthers) by one day. In contrast, CEPA application delayed the event by one day.

Application of CEPA, depressed the number of pollen grains in the anthers of the basal spikelets significantly, but its effect on the top spikelet was not significant (Figure 1). Application of the other chemicals (ethylene inhibitor) increased the survival of more pollen grains in the anthers of the basal spikelets. Their effect on the pollen number of the top spikelet was not significant. The total number of spikelets in the panicle was 140 (Sem ±5.17). CEPA application increased the number of barren spikelets, whereas the ethylene inhibitors improved the number of grain bearing spikelets in the panicle, compared to the control (Table 1).

3.2 *Dry weight of anthers*

The anthers of the top spikelet had a higher dry mass compared to the basal spikelets (Figure 2). In both top and basal spikelets, the dry weight of anthers increased steadily with time, but the rate was faster in the former, compared to the latter. The treatment with CEPA depressed the dry mass of the anthers of the basal spikelets, whereas the other chemicals improved their weight significantly (Table 2). The effect of the chemical treatments was not so discernible on the anthers of the top spikelets.

3.3 *Nonreducing sugar concentration of the anthers*

The nonreducing sugar concentration of the anthers declined with time in both top and basal spikelets (Figure 3). The sugar concentration was higher in the anthers of the basal spikelet compared to the top. Application of CEPA prevented the temporal decline in the concentration of nonreducing sugars in the basal spikelets (Table 2). But the other chemicals enhanced the rate of decline. The effects of the chemical treatments were not significant on the nonreducing sugar concentrations of the anthers of the top spikelets.

3.4 *Reducing sugar concentration of the anthers*

Compared to the nonreducing sugars, the temporal fluctuation in reducing sugar concentration was just the opposite (Figure 3). The concentration of reducing sugars increased in the anthers of the top and

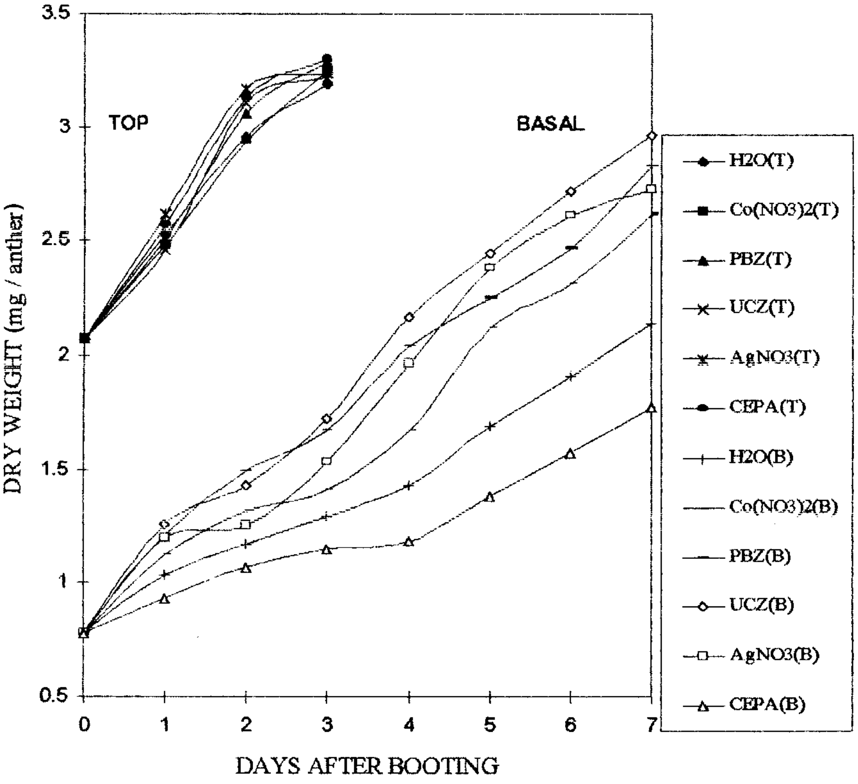

Figure 2. The effect of ethylene inhibitors ($(Co(NO_3)_2$, Paclobutrazol, Uniconazole and $AgNO_3$) and an ethylene releasing substance (CEPA) on dry weight of anthers of the top (T) and basal (B) spikelets.

basal spikelets with time. It reached a peak one day prior to anthesis and declined thereafter. The anthers of the top spikelet contained higher concentrations of reducing sugars compared to the basal. CEPA application decreased reducing sugar concentration, whereas the other chemicals increased it significantly in the anthers of the basal spikelets (Table 2). The treatments did not have any significant effect on the reducing sugar concentration of the anthers of the top spikelet. The temporal fluctuation in the concentration of total soluble sugars was similar to that of the reducing sugars.

3.5 *Starch concentration of the anthers*

The starch concentration of the anthers increased in the top and basal spikelets continuously with time; the former possessed higher concentrations of starch compared to the latter (Figure 3). CEPA application reduced the concentration significantly in the anthers of the basal spikelet, whereas application of the other chemicals raised it (Table 2). The chemical applications did not have any significant effect on the starch concentration of the anthers of the top spikelet.

3.6 *Acid invertase activity of the anthers*

Acid invertase activity in the anthers increased slowly with time, up to the stage just before anthesis, in both top and basal spikelets (Figure 4). In the following period, however, the activity of the enzyme increased rapidly, and was higher in the anthers of the top spikelets, compared to the basal. In the basal spikelets CEPA application depressed the activity of the enzyme, whereas the other chemicals increased it significantly (Table 2). The chemicals did not have a significant effect on the acid invertase activity of the anthers of the top spikelet.

3.7 *Sucrose synthase activity of the anthers*

The activity of sucrose synthase also increased with time in the anthers of both top and basal spikelets (Figure 4). The activity of the enzyme was higher in the anthers of the top spikelet, compared to the basal.

Table 2. F-values indicating influence of time and chemical treatments in dry weight, carbohydrate concentrations and activities of enzymes of anthers of rice cv. Lalat.

	CEPA		$Co(NO_3)_2$		$AgNO_3$		PBZ		UCZ	
	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
Dry weight (mg anther^{-1})										
Top	2.03^{NS}	0.07^{NS}	1.87^{NS}	0.00^{NS}	1.97^{NS}	0.08^{NS}	2.0^{NS}	0.00^{NS}	1.97^{NS}	0.01^{NS}
Basal	13.37**	7.66*	28.69***	12.43**	33.13***	30.68***	31.21***	37.50***	35.90***	56.76***
Reducing sugar (μg mg Fresh wt.$^{-1}$)										
Top	21.80*	0.04^{NS}	24.62*	0.20^{NS}	21.08*	0.06^{NS}	21.24*	0.07^{NS}	22.54*	0.01^{NS}
Basal	138.75***	529.78***	179.45***	241.88**	701.22**	1646.05***	610.44**	1122.71***	15.97***	49.40***
Non-reducing sugar (μg mg Fresh wt.$^{-1}$)										
Top	19.49*	0.01^{NS}	17.79*	0.42^{NS}	23.27*	0.62^{NS}	20.02*	0.28^{NS}	19.32*	1.00^{NS}
Basal	8.52**	11.00 *	19.75***	20.23**	20.81***	38.14***	21.41***	31.89***	22.80***	50.30***
Total soluble sugar (μg mg Fresh wt.$^{-1}$)										
Top	12.63*	0.04^{NS}	13.11*	0.38^{NS}	15.02*	0.00^{NS}	12.31*	0.00^{NS}	14.86*	0.04^{NS}
Basal	4.06*	13.28**	8.88**	18.45**	11.46**	42.04***	9.76**	25.37**	13.36**	55.61***
Starch (μg mg Fresh wt.$^{-1}$)										
Top	46.51**	0.34^{NS}	45.82**	0.47^{NS}	31.54**	2.54^{NS}	47.01**	0.02^{NS}	42.09**	0.10^{NS}
Basal	34.42***	25.75**	76.63***	27.03**	86.00***	79.31***	79.92***	58.79***	91.36***	128.34***
Sucrose synthase activity (μmol min^{-1}.gm Fresh wt.$^{-1}$)										
Top	10.68*	0.09^{NS}	12.18*	0.02^{NS}	15.14*	0.86^{NS}	12.12*	0.15^{NS}	12.12*	0.18^{NS}
Basal	9.74**	17.21**	15.18***	9.94*	20.68***	25.28**	16.24***	13.53**	19.99***	34.17***
Acid invertase activity (μmol min^{-1}gm Fresh wt.$^{-1}$)										
Top	31.91**	0.09^{NS}	30.12**	0.05^{NS}	36.30*	0.37^{NS}	28.87*	0.00^{NS}	30.63*	0.60^{NS}
Basal	60.11***	52.86***	94.81***	44.46***	94.91***	109.26***	73.88***	57.29***	103.91***	148.51***

Values are significantly different from the control at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ^{NS} = not significant using two way anova. F_1 = variation between days, F_2 = variation between treatments.

The effects of the chemical treatments were similar to that of acid invertase enzyme. However, per unit biomass of the anthers, the activity of acid invertase enzyme was much greater compared to that of sucrose synthase.

4. Discussion

Saini [32] stated that male reproductive organ development in plants is impaired when water stress coincides with meiotic or the anthesis stage of development, but the female gametophyte is relatively immune to the stress. Coincidence of stress at the pollen mother cell stage induces sterility of pollen grains [33] while exposure to stress at anthesis inhibits anther dehiscence and reduces pollen viability of rice [8, 9, 25]. Generally, stress-induced responses in plants are linked to the activity of ethylene or ABA. But experimental evidence in favour of either of the plant hormones causing impairment of male gametophyte development in rice is limited. In the present experiment, application of the ethylene synthesis inhibitors paclobutrazol [37], uniconazole [13] and cobalt [17, 19] and the ethylene action inhibitor silver [3] at late microsporogenesis stage [approximately 7 days before anthesis-33, 35] significantly improved pollen survival and anther development in the unproductive basal spikelets of rice panicles. In contrast, application of the ethylene-releasing substance CEPA [11] reduced pollen survival and depressed the number significantly. The positive and negative effects of these

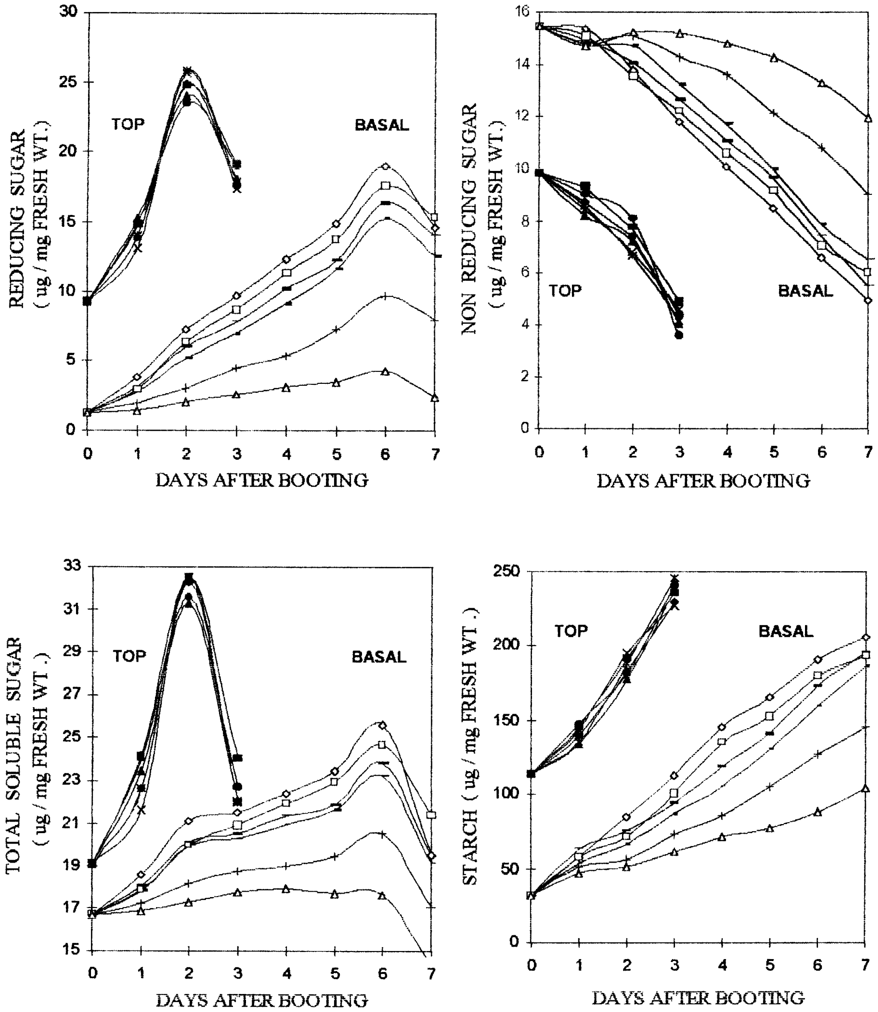

Figure 3. The effect of ethylene inhibitors and an ethylene releasing substance on the concentrations of carbohydrates of the anthers of top and basal spikelets. Symbols are similar to Figure 2.

chemicals on male gametophyte development corresponded to similar effects on grain setting of the spikelets. The ethylene inhibitors promoted grain setting and increased the number of grain bearing spikelets, whereas CEPA application increased the number of barren spikelets. This evidence suggests that endogenously produced ethylene from the panicle might be responsible for poor grain setting in the basal part of the panicle, because of its capacity for interference in male gametophyte development. However, direct effects of ethylene on fertilisation and seed development cannot be ruled out entirely. The immunity to the chemical treatments of the top spikelets may be due to either application of the chemicals at relatively later stage of pollen development, or higher efficiency of the male reproductive parts for development on such spikelets. Evidences in favour of either of these two propositions are lacking in rice. However, in maize, chemical treatments which induce ethylene synthesis have been reported to cause abortion of younger apical kernels, while older kernels on the basal region of the panicle are not adversely influenced at the same time [6].

Wada et al. [35] observed that seven days before heading in rice, vacuolated pollen containing generative and vegetative cells were derived from the microspores. The generative cell divides mitotically again to produce two sperm cells a couple of days

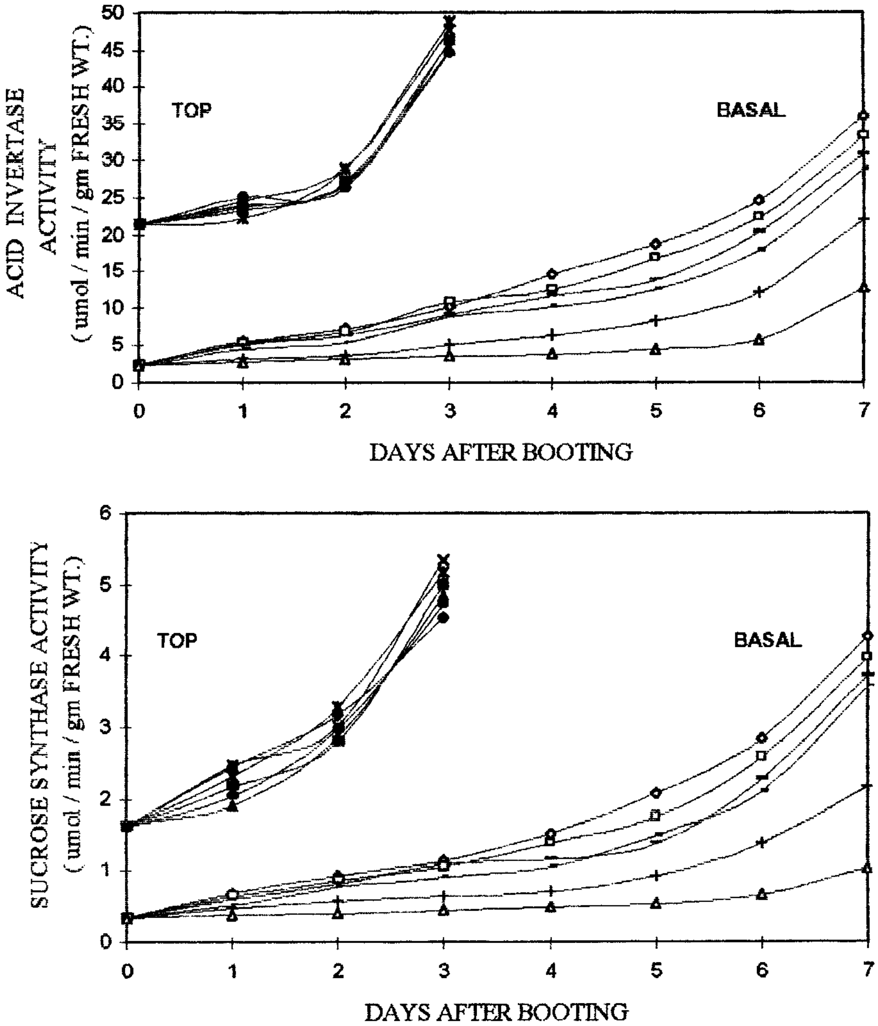

Figure 4. The effect of ethylene inhibitors and an ethylene releasing substance on the activities of invertase and sucrose synthase enzymes of the anthers of top and basal spikelets of rice cv. Lalat. Symbols are similar to Figure 2.

later. While mitotic divisions are taking place in the microspore nuclei, the pollen accumulated a large amount of starch and proteinaceous materials in the cytoplasm and vacuole at the cost of removal of water. It is suggested [33] that impairment of starch synthesis can have a causal role in the induction of pollen sterility of rice when subjected to water stress. The present investigation supports these suggestions as starch concentration of the poorly formed anthers of basal spikelet was found to be lower compared to the well developed anthers of the top spikelet. Inadequate synthesis of starch in the anthers of the basal spikelet resulted in the poor consumption of nonreducing sugars and consequent decline in the dry matter accumulation rate. In contrast, male gametophyte development was adequate in the top spikelet; the anthers possessed a higher biomass and starch accumulation rate. Consequently, the pool of nonreducing sugar was consumed rapidly. Adequate synthesis of starch might have helped in the survival of a larger number of pollen grains in the anthers.

Jenner [14] proposed that partitioning of sugars into starch reserves is regulated by assimilate supply from the source organs and demand from the sink. In the present experiment, the anthers of the basal spikelet accumulated more nonreducing and less reducing sugars compared to the top. Ethylene inhibitor applications, which promoted anther development on

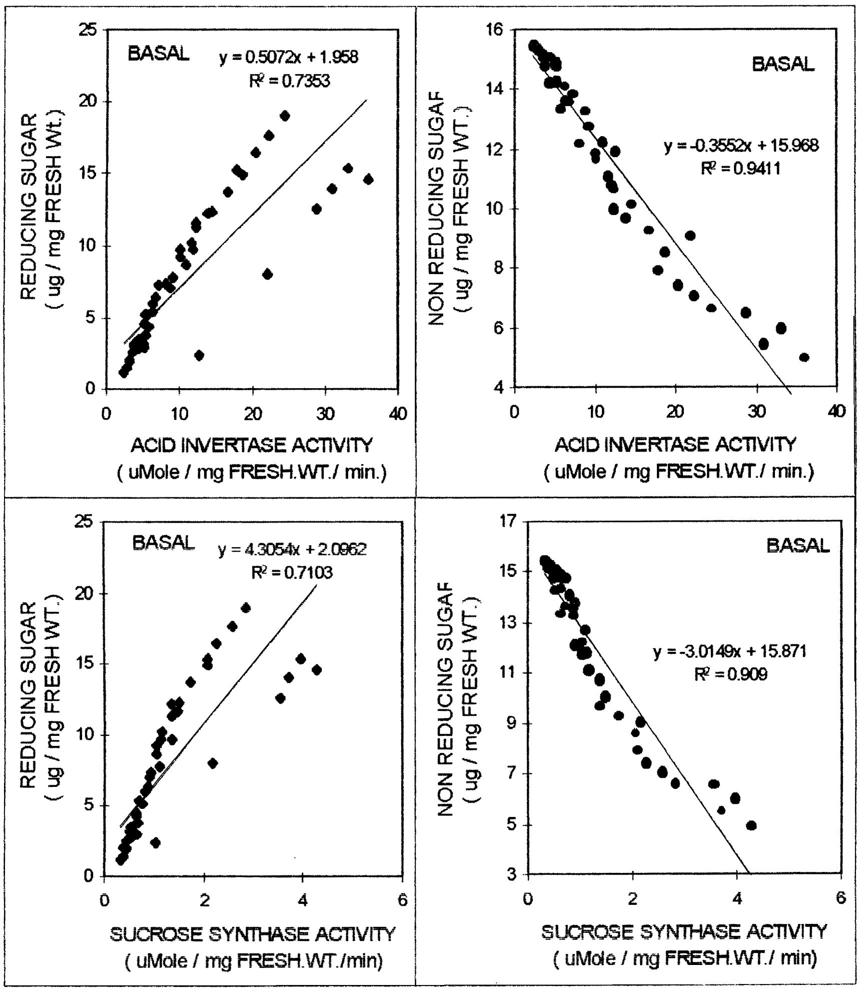

Figure 5. Correlation between sugar concentration and activities of enzymes sucrose synthase and invertase in the anthers of basal spikelets of rice cv. Lalat.

the basal spikelet, also reduced the concentration of nonreducing sugars and enhanced the concentration of reducing sugars. This evidence suggests poor sink efficiency of the anthers on the basal spikelet for utilisation of sugars. CEPA application, which depressed starch concentration of the anthers, increased accumulation of nonreducing sugars. This observation also supports the proposition that accumulation of nonreducing sugars is correlated with lack of demand and utilisation and failure of anther development in the basal spikelet is unrelated to any bottleneck in the supply of assimilates.

Sucrose is the principal form of sugar translocated in the sink organs of rice [28]. It enters into the starch synthesis pathway in two different routes primed by the enzymes sucrose synthase and invertase [23, 29]. In the present experiment, the activities of both the enzymes showed a positive correlation with concentration of reducing sugars and a negative correlation with the concentration of nonreducing sugars (Figure 5). Although both enzymes are responsible for the utilisation of sucrose and synthesis of starch in the present system, higher activity of invertase indicates its dominance over that of sucrose synthase. Thus, poor activities of the enzymes (mainly invertase) reduced sink efficiency of the anthers of the basal spikelet for starch synthesis and resulted in the accumulation of nonreducing sugars. Higher activities of the enzymes increased the pool of reducing sugars in the anthers of the top or ethylene inhibitor treated basal spikelet. Under such conditions the pool of nonreducing sugars depleted. CEPA treatment depressed the activities of the enzymes and reduced the efficiency for starch synthesis. These results are comparable to those of Sheoran and Saini [33] and Dorion et al. [7], where water stress inhibited activity of acid invertase in the anthers of rice and wheat, respectively, and resulted in poor synthesis of starch and pollen abortion. Similarly, in faba bean, invertase activity has been reported to contribute in the establishment of sink strength of young seeds regulating hydrolysis of sucrose [38].

The results of the present experiment show that it is unlikely that pollen development in the anthers of rice is inhibited due to carbohydrate starvation during the pollen mitosis phase. Instead, a cause and effect relationship between starch synthesis and pollen development seems more probable. In the starch synthesis pathway, acid invertase enzyme plays a dominant role and regulates the conversion of nonreducing sugar (sucrose ?) into reducing sugars for their subsequent condensation to starch. The application of ethylene inhibitors and CEPA suggested that correlative inhibition of spikelet development in the rice panicle is controlled by hormone(s), and ethylene performs a vital role in the mechanism. Net development of a plant organ is influenced by the ratio between promotive and inhibitory hormones, and suppression of ethylene might have prompted growth stimulating hormones, like gibberellins and cytokinins, to increase growth and development of the floral organs of the basal spikelets [27]. In contrast, enhancing the formation of ethylene by application of CEPA might have stimulated accumulation of ABA [12], or breakdown of cytokinin [4]. The resulting decline in the ratio between promotive to inhibitory hormones can induce poor development of the male gametophyte and grain setting. Although rice anthers have not been assayed for ABA, in wheat it is reported that anthers of the inferior florets located on the distal position of a spikelet branch possess a higher concentration of ABA, in comparison to the superior florets on the proximal position [18].

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