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Ascorbic Acid Priming Enhances Seed Germination and Seedling Growth of Winter Wheat under Low Temperature Due to Late Sowing in Pakistan

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Abstract: Poor seed germination is a crucial yield-limiting factor when winter wheat is sown under low temperature. The objective of this study was to evaluate the role of ascorbic acid (AsA) in the extenuation of the harmful effects of low temperature at early and reproductive stages of wheat during 2016–2017 (15 November to 15 December). A two-year experiment was conducted using a randomized complete block design with split plot arrangement and with three replicates. Sowing dates (15 November and 15 December) were allotted to the main plot while seed priming (control, hydro-priming, and AsA priming) were allotted to the sub-plot. Results demonstrated that AsA priming significantly boosted different yield characteristics including chlorophyll content, tillers per unit area, number of grains per spike, and 1000-grain weight, contributing higher productivity and biomass during 2016–2017. The results further revealed that AsA could induce the up-regulation of diverse antioxidants (super oxide dismutase (SOD), peroxidase (POD), and catalase (CAT)), thus offsetting the adverse effects of sub-supra optimum temperatures of late sowing wheat. It is therefore concluded in this work that AsA priming enhances stand establishment, yield and yield-related traits, antioxidant enzyme activities, and chlorophyll contents when wheat is sown under low temperature.

Keywords: antioxidant capacity; ascorbic acid; germination enhancement; hydro-priming; yield; wheat

1. Introduction

As a staple food and rich source of carbohydrates after corn and rice, wheat ranks as the third-largest consumed crop in the world. Similarly, it is the major staple food in Pakistan with an annual cultivated area of 9,052,000 hectares and with a production of 25.75 million tonnes. Wheat makes an indispensable contribution to the daily dietary intake (it makes up 60% of calories) of an ordinary person and this is why it monopolizes sovereign agricultural policies at the governmental and developmental levels [1]. The irrigated savanna of Pakistan is adapted to cotton-wheat and rice-wheat cropping patterns which

are located from the valley of Peshawar to that of the Indus valleys of Punjab and Sindh. In both of the cropping patterns, almost more than 70% of the area is covered by Rabi, a wheat crop. The delay in the maturation of basmati cultivars of rice and that of the picking of cotton varieties results in the late sowing of wheat up to the middle of December [2]. The adequate and optimal time for the sowing of wheat and potential production starts from late October through to the middle of November. The yield is reduced by $50 \text{ kg ha}^{-1} \text{ day}^{-1}$ because of the delay to wheat sowing after the middle of November [3]. The reduction in the wheat yield is due to the meager plant population that may occur as a result of scarce and inconsistent germination due to low temperatures. Wheat is a photosensitive crop in nature that accomplishes its life cycle (growth and development) in a short specific duration, and as a result of this, the plant may not be able to accumulate starch because it does not rely on sowing time [4]. Environmental and economic benefits in agriculture could be attained with a fast and homogeneous mixture which may result in a higher degree of automation, apparent weed control, and disease pressure consolidation in the field, and provide relief to imminent precision farm technologies.

A set of certain physio-chemical, biological, and integrated mechanisms play their roles in overcoming losses in the yield crop [5]. Amongst these mechanisms, the invigoration of seeds with certain chemical agents and nutrients is one of the most feasible approaches to acclimatizing yield losses [1]. These invigorating mechanisms comprise hydropriming, osmoconditioning, osmohardening, hardening, hormonal, and vitamin priming. Seed priming is an imperative approach related to the progression of seed germination. It has been extensively adapted to synchronize and enhance germination and seedling growth [1,6]. The priming of seeds amends the performance by accelerating homogeneous germination via standard and efficient seedlings in various crops which have impartial agronomic elaborations even under detrimental germination environments [7,8]. This is the reason for cutting back the sensitivity of exotic factors and privileging the development of seedlings in extensive agro-climatic environments [7,9].

Plant growth regulators and hormones boost the performance of various crops through priming and pre-sowing mechanisms [8]. Enzymes have certain specific roles related to their use within priming agents; ascorbic acid ameliorates the final emergence percentage (FEP) and emergence index (EI) while reducing mean emergence time (MET) and time to 50% emergence (E50) in wheat [1]. Ascorbic acid (AsA) executes as a co-factor for certain enzymes and maintains the process of phytohormone mediating signaling [10] and several plant physiological processes [11]. AsA also controls cell division and cell expansion, modulates plant sense, and is involved in photosynthesis, hormone biosynthesis, and regeneration of antioxidants. AsA restrains the formation of tocopherol, which helps plants to defend themselves against many environmental hazards and stresses [12]. AsA impacts cell division and cell elongation in plants [13]. It has a role in the process of phytohormone-mediated signaling when the plants are shifting from vegetative to the reproductive phase and in the ultimate stage of development and senescence [10]. As a pre-sowing invigorating agent, AsA can enhance plant mechanisms to cope with abiotic stress. Ascorbic acid used as a pre-sowing seed treatment improves abiotic stress tolerance in wheat. Many researchers have used ascorbic acid for the mitigation of abiotic stresses. However, little is known about the use of ascorbic acid to improve seed germination under low temperatures due to late sowing. The current study aims to explore the potential role of AsA priming and hydro-priming on stand establishment, biochemical traits, and yield and yield-related traits under late sowing conditions in the field.

2. Materials and Methods

2.1. Seed Priming Agent

Ascorbic acid (L+ascorbic acid) was purchased from Sigma AldrichTM (www.sigmaaldrich.com) with the catalog number A106755-1g/487623. Prior to conducting the experiment, the best ascorbic acid priming concentrations (10, 20, 30, 40, 50, and 60 AsA mg L^{-1}) were optimized separately. According

to the results, the best concentration of ascorbic acid priming was with 50 mg L⁻¹ (data not shown). The seeds were soaked for eight hours by maintaining 1:5 (w/v) seed to solution ratio [14].

2.2. Plant Materials

Seeds of common sown cultivar Pirsabak-2013 originated from Cereal Crops Research Institute, Pirsabak, Nowshera, Pakistan with a germination rate of $\geq 90\%$ were used in this study. Healthy, unbroken, and spotless seeds were sorted out manually, afterward; the selected seeds were subjected to sterilization using sodium hypochlorite (3% v/v for 10 minutes) and then thoroughly washed with distilled water. The sterilized seeds were dried using blotting paper and further drying was done with air dry at room temperature to reach the initial weight.

2.3. Experimental Details

For the evaluation of seed priming in extenuating the harmful effects of late sowing, seeds were subjected to hydro-priming and ascorbic acid priming however untreated seeds were taken as control. During priming, seeds were soaked in distilled water with 1:5 (w/v) ratios for eight hours. After pre-experimenting for the best concentration of ascorbic acid for seed priming, the 50 mg L⁻¹ AsA was approved and used for seed priming. Wheat seeds were soaked in an aerated solution of ascorbic acid (50 mg L⁻¹) for eight hours with gentle constant agitation at 25 °C. The ratio of 1:5 seed to water was used. After priming treatments seeds were given three surface washes with distilled water and re-dried near to their original weight with forced air under the shade at 25 ± 3 °C. After priming treatments, treated seeds along with dry seed (control) were sown under normal (15 November) and late (15 December) sown conditions. The soil analysis and climatic data during both years were also recorded (Figure S1; Table S1).

2.4. Crop Husbandry

The proposed two-year-study was conducted at Agricultural Research Station, Harichand, Charsadda, Pakistan (34.14 latitudes North and 71.73 longitude East and it is situated at 303 meters above sea level) to evaluate seed priming impacts on normal and late sown wheat. The net sizes of subplots were 6 m × 1.8 m. Pre-soaking irrigation was applied before seedbed preparation. Seedbed was prepared by two cultivations with a tractor-mounted cultivator each followed by planking. The crop was sown with the help of a single row hand drill using 130 kg seed per hectare. Nitrogen, phosphorus, and potash were applied at rate a of 120, 90 and 60 kg ha⁻¹. Urea, Diammonium phosphate, and Sulphate of potash were the sources of nitrogen, phosphorus, and potassium fertilizers, respectively. Phosphorus and potassium fertilizers were applied as a basal dose. However, Urea was applied in three equal splits viz. at sowing, first irrigation, and second irrigation. Four irrigations, each of three-acre inches, were applied at critical growth stages of wheat crop.

2.5. Plant Survey

2.5.1. Stand Establishment Traits

After seed sowing, the experiment was visited daily and final germination was counted until a constant count was achieved. Data concerning the emergence and seedling vigor traits were collected according to ISTA protocols [15]. Mean emergence time (MET) was calculated according to the equation of Ellis and Robert [16].

$$\text{MET} = \Sigma Dn / \Sigma n \quad (1)$$

where n is the number of seedlings, which were emerged on day D, and D is the number of days counted from the start of seedling emergence.

Time to 50% emergence (E50) was calculated according to Farooq et al. [17]

$$[E50 = t_i + \{(N/2 - n_i)/(n_j - n_i)\} \times (t_j - t_i)] \quad (2)$$

where N is the number of final emergence count and n_i, n_j cumulative number of seeds emerged at adjacent days t_i and t_j when $n_i < (N + 1)/2 < n_j$.

The coefficient of uniformity of emergence (CUE) was calculated using the following formulae of Bewley and Black [18].

$$CUE = \sum n / \sum [(t - t)^2 \times n] \quad (3)$$

2.5.2. Biochemical Traits

At the booting stage, flag leaf (0.5 g) was dipped overnight with 5 mL 80% acetone at 0–4 °C. The extracts were centrifuged at $10,000 \times g$ for 5 min. The absorbance of the supernatant was read at 645, 663 and 480 nm using a spectrophotometer (Hitachi-U2001, Tokyo, Japan). Chlorophylls a and b were determined by the formula described by Arnon [19].

$$Chl\ a = [12.7 (OD\ 663) - 2.69 (OD\ 645)] \times V/1000 \times W \quad (4)$$

$$Chl\ b = [22.9 (OD\ 645) - 4.68 (OD\ 663)] \times V/1000 \times W \quad (5)$$

where V = volume of the extract (mL), W = weight of the fresh leaf tissue (g)

For enzymatic antioxidants determination, extraction of leaf sample was done in 5 ml of 50 mM phosphate buffer (pH 7.8), after centrifugation at $15,000 \times g$ for 20 min, the supernatant was used in further assay for SOD (super oxide dismutase) activity [20], CAT (catalase) and POD (peroxidase) activity [21] by recoding absorbance at 560, 240, and 470 nm, respectively.

SOD activity inhibits photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The monitoring of this inhibition is used to assay SOD activity. Reaction mixture was prepared by taking 50 μ L enzyme extract and adding 1 mL NBT (50 μ M), 500 μ L methionine (13 mM), 1 mL riboflavin (1.3 μ M), 950 μ L (50 mM) phosphate buffer, and 500 μ L EDTA (75 mM). This reaction was started by keeping the reaction solution under 30 W fluorescent lamp illuminations and turning the fluorescent lamp on. The reaction stopped when the lamp turned off 5 min later. The NBT photoreduction produced blue formazane which was used to measure the increase in absorbance at 560 nm. The same reaction mixtures without enzyme extract in dark were used as blank. The SOD activity was determined and expressed as SOD IU $\text{min}^{-1} \text{mg}^{-1}$ protein [20].

CAT activity assayed by decomposition of H_2O_2 and change in absorbance due to H_2O_2 was observed every 30 s for 5 min at 240 nm using a UV-visible spectrophotometer. The reaction mixture for CAT contained 900 μ L H_2O_2 (5.9 mM) and 2 mL phosphate buffer (50 mM). The reaction was started by adding 100 μ L enzyme extract to the reaction mixture. The Catalase activity was expressed as $\mu\text{mol of } \text{H}_2\text{O}_2 \text{ min}^{-1} \text{mg protein}^{-1}$ [21].

Activities of POD were assayed following Chance and Maehly [21] with some modification. The activity of POD was determined by the guaiacol oxidation method. The final volume of the reaction mixture for POD (3 mL) contained 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 40 mM H_2O_2 , and 0.1 mL enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined every 20 sec. One unit POD activity was defined as the change of 0.01 absorbance unit per min per mg of protein.

2.5.3. Yield and Yield Related Traits

At the final harvest, the number of tillers was counted from a unit area in each plot. Plant height was measured using a meter rod. Grains from the ten randomly selected spikes were threshed and counted separately. Then an average number of grains per spike was calculated. The 1000-grain weight was recorded by an electric balance. For the determination of biological yield, an area of 1m^2 was harvested from each plot at the fully mature stage. Total wheat biomass was measured by using a weighing balance. The grains obtained from each plot after threshing was weighed by a weighing balance to record the grain yield.

2.6. Statistical analysis

The experiments were carried out in a randomized complete block design using a split-plot arrangement having three replications. Main plot factors owing dates and subplot factor seed priming techniques. The collected data were analyzed using Fisher's analysis of variance technique with Software Statistix 8.1; least significance difference (LSD) was used to test the significance among treatments at 0.05 significance level in all cases [22]. Microsoft Excel 2010 was used for plotting graphs.

3. Results

3.1. Stand Establishment Traits

All priming treatments (hydro and AsA priming) had a significant effect on mean emergence time (Table 1). The growing season's variation showed that sowing conditions had an obvious impact on germination time. More days were taken during the second year (2017). A significant interaction was revealed among priming treatments and sowing dates for germination time during the first year (2016) (Figure 1). Hydro-primed seeds resulted in quicker emergence which was statistically at par with AsA primed seeds. Unprimed (control) seeds took more days when sown late (15, December). Significantly lowered mean emergence time was observed in AsA primed seeds over other treatments. Nevertheless, quicker emergence pattern was observed in early sown (15, November) seeds in response to applied treatments during the second year while late sown seeds resulted in erratic emergence.

Table 1. Mean comparison of the effect of seed priming and sowing dates on stand establishment of wheat.

Years	Mean Emergence Time (days)	Time to 50% Emergence (days)	Co-efficient of Uniformity Emergence (CUE)	Final Germination Count
2016	10.77	6.16	0.78	208.9
2017	12.37	8.64	0.66	182.7
LSD	0.37	0.62	0.07	14.86
Sowing Dates (SD)				
15-November	7.69	4.94	0.74	204.1
15-December	16.10	11.34	0.57	161.4
LSD	1.44	0.95	0.26	9.16
Priming Techniques (P)				
Control	12.88 a	9.51 a	0.52 b	152.4 c
Hydro-priming	12.46 a	8.57 b	0.63 b	171.4 b
AsA priming	11.76 b	7.83 c	0.81 a	224.4 a
LSD	0.43	0.79	0.25	13.86
Interaction				
SD × P (2016)	* (Figure 1)	* (Figure 2)	ns	* (Figure 3a)
SD × P (2017)	Ns	ns	ns	* (Figure 3b)

Means not sharing same letter (s) differ significantly at $p \leq 0.05$. ns: non-significant, * = Significant at $p \leq 0.05$.

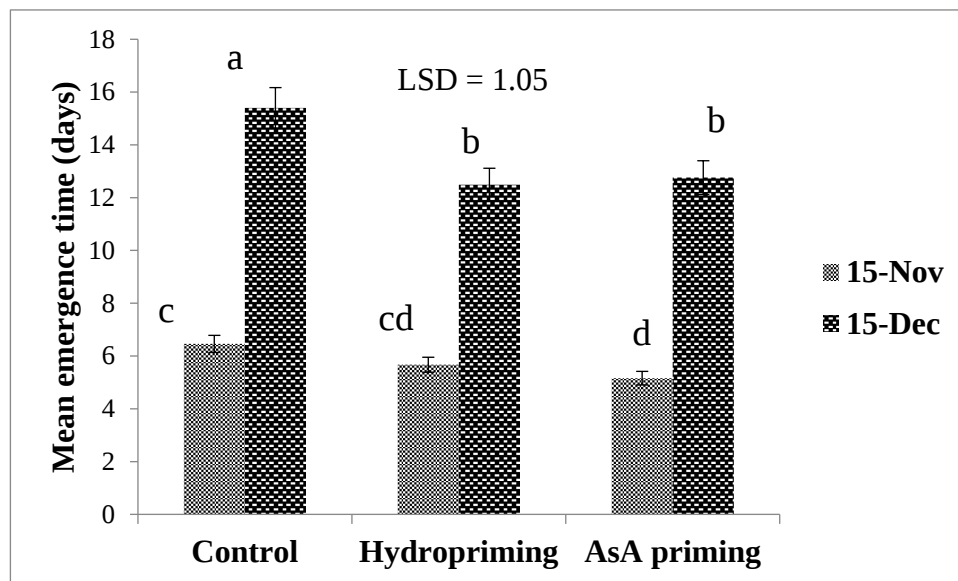


Figure 1. Interaction between priming techniques and sowing dates for mean emergence time (days) during 2016. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

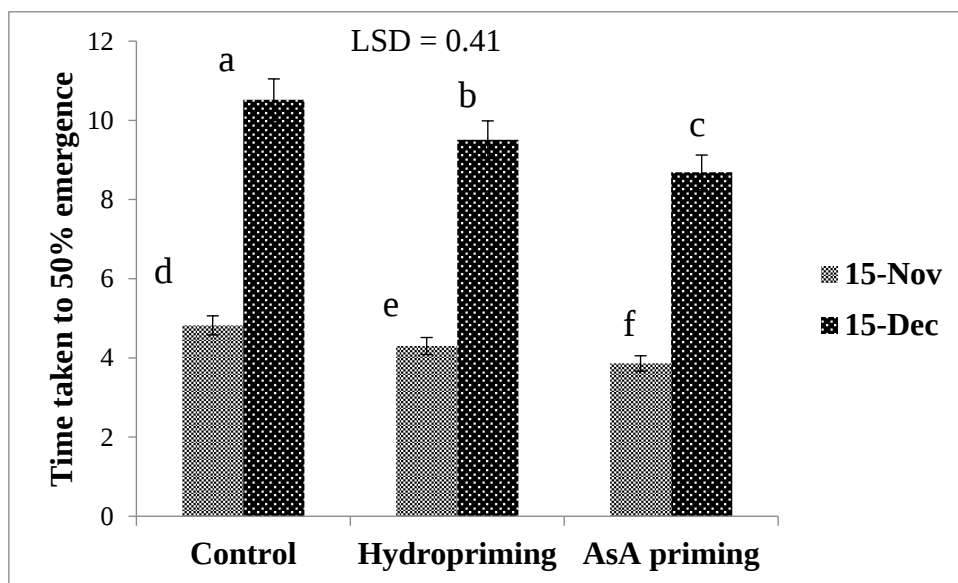


Figure 2. Interaction between priming techniques and sowing dates for time taken to 50% emergence during 2016. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

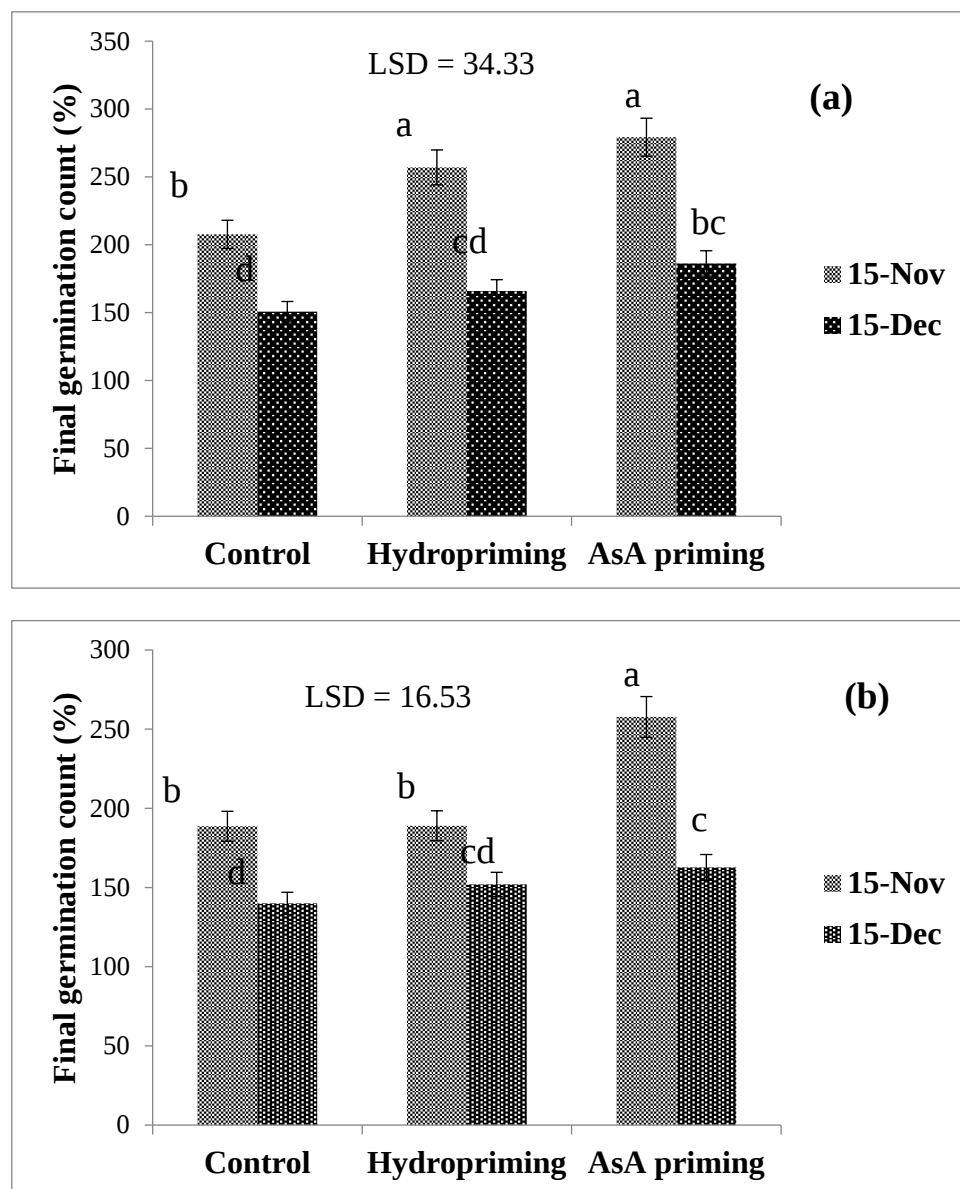


Figure 3. (a, b) Interaction between priming techniques and sowing dates for final germination count during 2016-17. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

Time taken to 50% germination (T_{50}) was significantly influenced by priming treatments under both normal and late sown conditions (Table 1). Fluctuating patterns of the environment with growing seasons significantly affected T_{50} under both sowing conditions. The maximum time for 50% emergence was taken during the second year. Interaction between priming treatments and sowing dates was found significant during the first year (Figure 2). Unprimed (control) seeds took more time for 50% emergence under late sown conditions. AsA primed seeds took less time for 50% emergence under normal sown condition. During the first year, AsA primed seeds took less time for 50% emergence under both sowing conditions followed by hydro-primed seeds over unprimed seeds. In late sown conditions time taken to 50% emergence was higher during the second year.

Data depicts that the impact of sowing dates and priming treatments was significant on the coefficient of uniformity emergence (CUE) during both years (Table 1). CUE was higher during the first year than the second year. Interaction between sowing dates and priming treatments was non-significant during both years. Late sown seeds exhibited lower CUE. AsA primed seeds exhibited

higher CUE followed by hydro-primed seeds as compared to unprimed seeds (control) during both years.

Priming treatments significantly affect final germination count under normal and late sown conditions during both years (Table 1). During both years germination count was more. Sowing dates and priming interaction was found significant during both years (Figure 3a,b). Priming treatments resulted in more plants under normal conditions during the first year. Maximum germination count was observed in AsA primed seeds while the minimum germination count was observed in unprimed seeds under late sown conditions.

3.2. Chlorophyll Contents in Flag Leaves

Data analysis revealed that priming treatments and sowing dates have a significant effect on chlorophyll a content (Table 2). Varied climatic conditions showed a considerable effect on chlorophyll a content during the two-year study. During the first year, more chlorophyll content was measured. Normal sowing resulted in more chlorophyll a content than late sowing during both years. Priming treatments have a positive effect on chlorophyll a content under normal and late sown conditions. AsA primed seeds gave more chlorophyll a content than unprimed (control) seeds. Priming treatments and sowing dates interaction were significant during the second year while insignificant during the first year. AsA primed seeds grown under normal condition resulted in more chlorophyll a content while lower chlorophyll a content was recorded in unprimed (control) seeds under late sown condition (Figure 4).

Table 2. Mean comparison of the effect of seed priming and sowing dates on biochemical traits of wheat.

Years	Chlorophyll a (mg g ⁻¹)	Chlorophyll b (mg g ⁻¹)	Peroxidase (unit mg protein ⁻¹)	Catalase (unit mg protein ⁻¹)	Super Oxide Dismutase (unit mg protein ⁻¹)
2016	2.09	0.718	8.88	14.13	137.2
2017	1.90	0.613	7.27	10.32	121.6
LSD	0.119	0.154	0.62	3.066	7.37
Sowing Dates (SD)					
15-November	2.24	0.693	4.81	11.83	125.9
15-December	2.13	0.533	9.73	8.80	148.4
LSD	0.22	0.255	1.35	2.51	3.09
Priming Techniques (P)					
Control	1.04 c	0.551 c	5.93 c	8.79 b	129.2 c
Hydropriming	2.21 b	0.621 b	6.87 b	9.97 b	136.9 b
AsA priming	2.23 a	0.667 a	8.03 a	12.18 a	145.5 a
LSD	0.17	0.147	0.63	2.37	4.72
Interaction					
SD × P (2016)	ns	ns	* (Figure 5a)	ns	ns
SD × P (2017)	* (Figure 4)	ns	* (Figure 5b)	ns	* (Figure 6)

Means not sharing same letter (s) differ significantly at $p \leq 0.05$. ns: non-significant, * = Significant at $p \leq 0.05$.

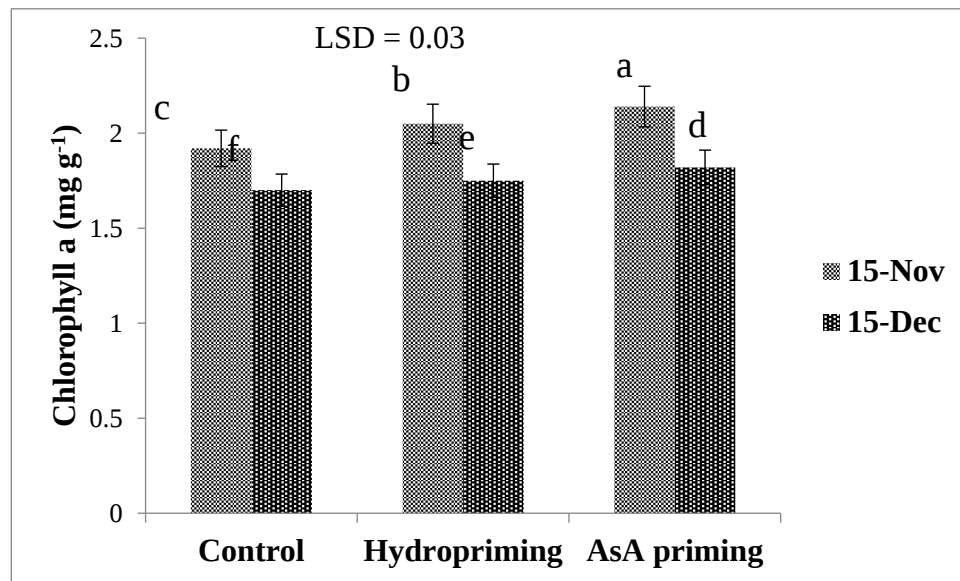


Figure 4. Interaction between priming techniques and sowing dates for chlorophyll a during 2017. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

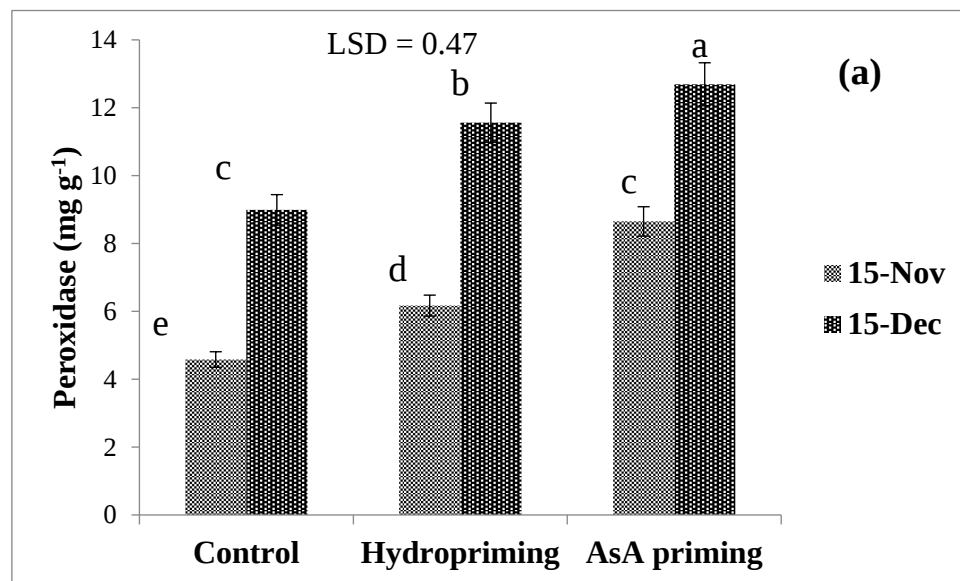


Figure 5. Cont.

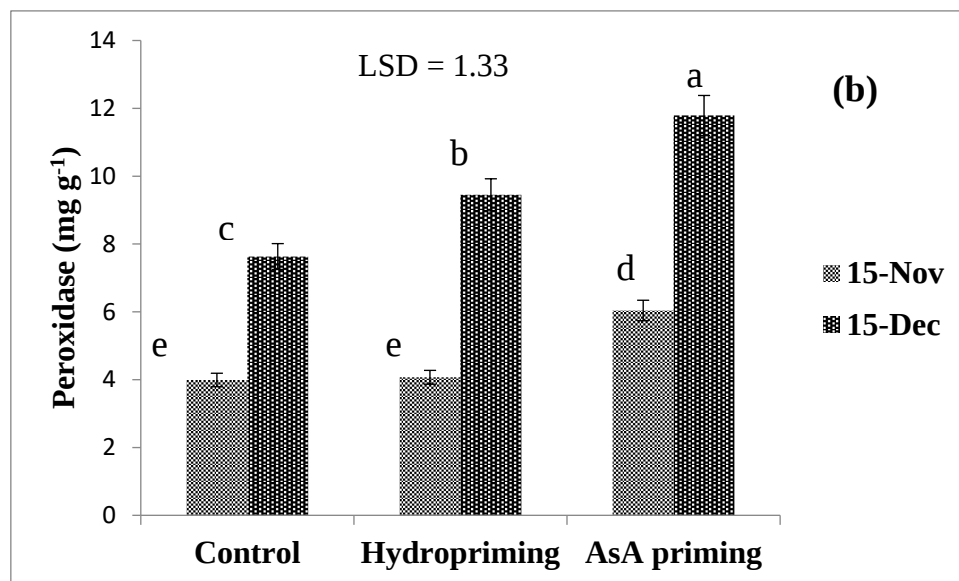


Figure 5. (a, b) Interaction between priming techniques and sowing dates for peroxidase during 2016-17. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

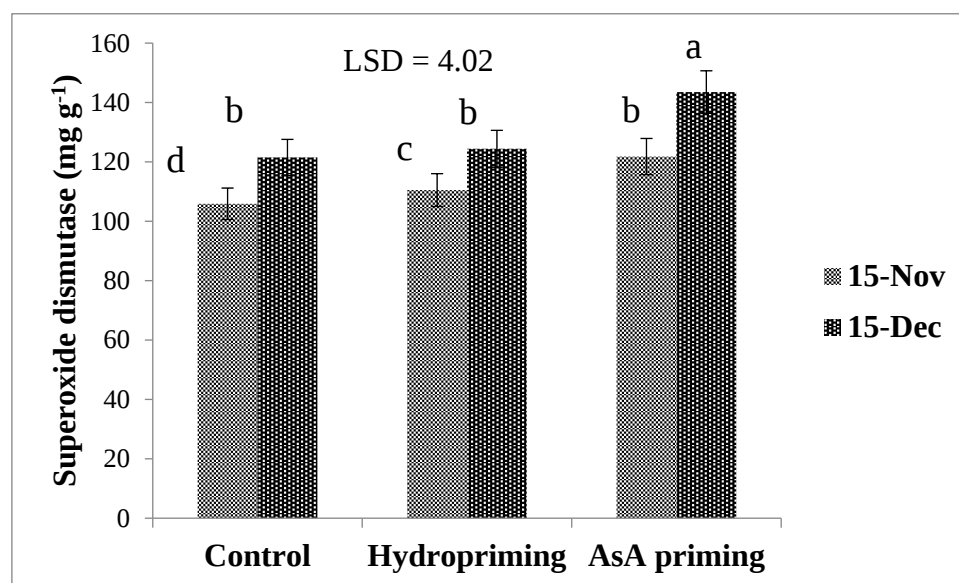


Figure 6. Interaction between priming techniques and sowing dates for superoxide dismutase during 2017. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

Analyzed data exhibited that diverse climatic conditions along with crop seasons and priming treatments have a prominent impact on chlorophyll b content (Table 2). Higher chlorophyll b content was recorded during the first year than the second year. Varied sowing dates adversely affect chlorophyll b content as late sown seeds resulted in lowered chlorophyll b content than early sown seeds. Hence, more chlorophyll b content was noted in early sown seeds compared with late sown seeds. Concerning priming treatments, AsA primed seeds gave more chlorophyll b content than unprimed (control) seeds. Interactive effect among priming treatment and sowing dates were found non-significant during both years. Data collected for chlorophyll b content during the second year exhibited the same trend as for the first year.

3.3. Antioxidant system in flag leaves

Data regarding POD activity revealed that different sowing dates and priming treatments had a considerable impact during both years (Table 2). Year effect on POD activity illustrates that POD activity was higher during the first year than the second year. Varied sowing dates and priming treatment interaction was also found to be significant during both years. POD activity was higher under the late sowing condition than the early sowing conditions. POD profile was also affected by priming treatments. AsA primed seeds gave higher POD activity followed by hydro-primed seeds and unprimed (control) seed under late sown condition. Priming treatments have a prominent and more noticeable impact on the POD profile under late sown condition (Figure 5a,b). Both years have the same trend for POD concerning varied sowing dates and priming treatments.

Data exhibited that priming treatments and sowing dates have a remarkable impact on CAT activity during both years (Table 2). The CAT activity was higher during the first year than the second year. The CAT activity was affected by sowing dates as early sowing resulted in higher CAT activity compared to late sowing. CAT activity increased in plant tissues due to priming. AsA primed seeds gave higher CAT activity followed by hydro-primed seeds while unprimed (control) seeds resulted in lower CAT activity. CAT activity during the second year exhibited the same pattern as the first year. The interactive effect among both factors was found insignificant during both years.

Priming treatments and varied sowing date significantly influenced during both years (Table 2). Year effects showed higher SOD content during the first year than the second year. Late sown seeds resulted in higher SOD content than early sown seeds. AsA primed seeds resulted in higher SOD content during the first year than unprimed (control) seeds. However interactive effect among priming treatments and sowing dates were found insignificant during the first year and were significant during the second year. The interactive effect depicted that AsA primed seeds gave higher SOD content under late sown condition while unprimed (control) seeds gave lower SOD content under early sown condition (Figure 6).

3.4. Grain Yield and Yield Related Traits

Data showed significant variation in the number of tillers due to priming treatments and sowing dates during both years (Table 3). During the first year, the maximum number of tillers was observed. Normal sowing showed maximum number of tillers per unit area over late sowing. The interactive effect was found significant during both years (Figure 7a,b). AsA primed seeds resulted in more tillers under normal sowing. Unprimed (control) seeds exhibited less number of tillers under late sown conditions. During the second year, the same response was observed.

Table 3. Mean comparison of the effect of seed priming and sowing dates on yield and yield traits of wheat. In the formatted paper this table was missing so I insert it.

Years	Tillers Per Unit Area (m ⁻²)	Plant Height (cm)	Grain Number	1000-Grain Weight (g)	Grain Yield (t ha ⁻¹)	Biological Yield (t ha ⁻¹)
2016	345.7	94.8	43.5	35.7	4.0	14.2
2017	335.5	88.8	41.3	34.4	4.8	12.6
LSD	10.85	5.36	0.96	0.66	0.3	0.63
Sowing Dates (SD)						
15-November	376.7	97.1	45.7	38.0	5.0	16.7
15-December	294.3	80.5	36.8	29.9	3.4	11.8

Table 3. Cont.

Years	Tillers Per Unit Area (m ⁻²)	Plant Height (cm)	Grain Number	1000-Grain Weight (g)	Grain Yield (t ha ⁻¹)	Biological Yield (t ha ⁻¹)
LSD	72.91	6.41	4.42	2.63	0.47	2.53
Priming Techniques (P)						
Control	307.2 c	81.0 b	38.6 b	31.7 c	4.2 c	13.2 c
Hydropriming	337.7 b	90.7 a	41.3 ab	34.6 b	4.8 b	14.3 b
AsA priming	360.9 a	93.8 a	43.9 a	36.9 a	5.2 a	15.1 a
LSD	9.66	7.97	4.26	2.11	0.43	0.82
Interaction						
SD × P (2016)	* (Figure 7a)	ns	ns	* (Figure 8)	* (Figure 9)	ns
SD × P (2017)	* (Figure 7b)	ns	ns	ns	ns	* (Figure 10)

Means not sharing same letter (s) differ significantly at $p \leq 0.05$. ns: non-significant, * = Significant at $p \leq 0.05$.

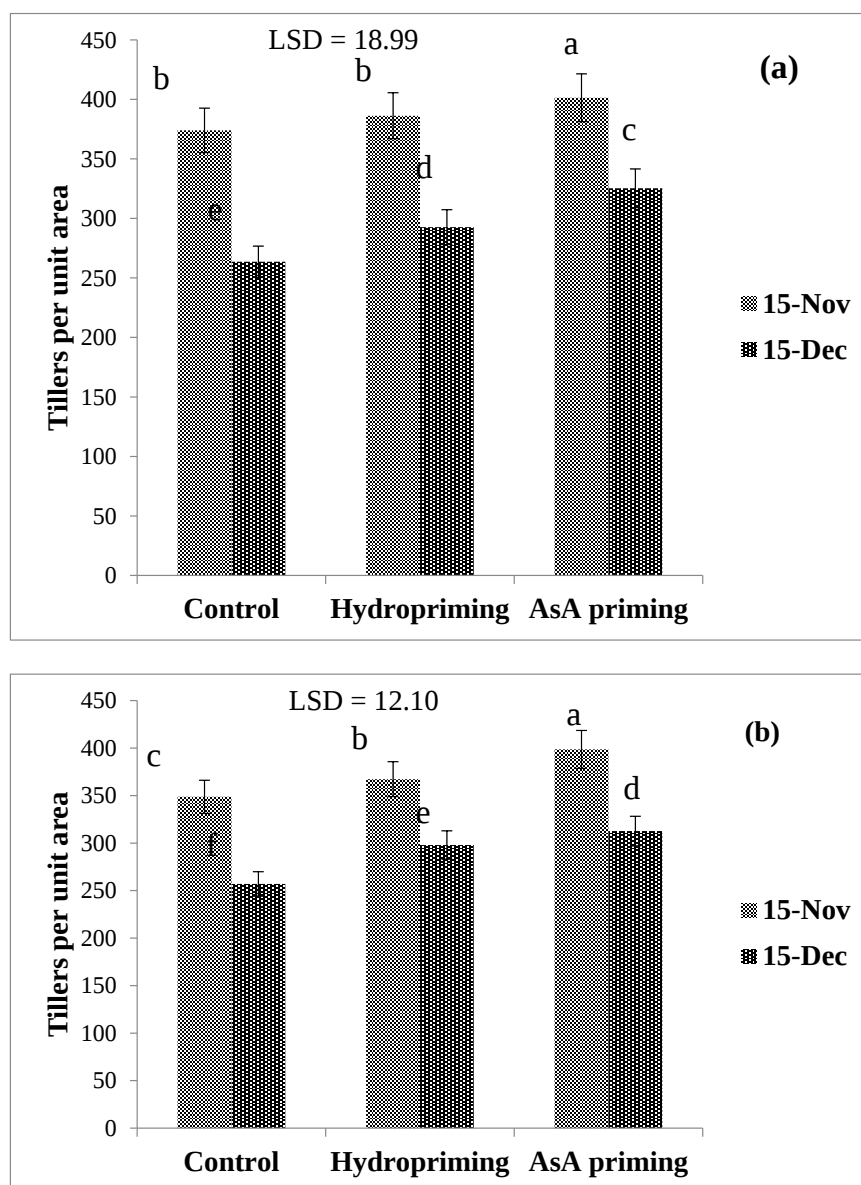


Figure 7. (a, b) Interaction between priming techniques and sowing dates for tillers per unit area during 2016-17. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

Plant height was significantly influenced by priming treatments and sowing date during both years (Table 3). Taller plants were observed during the first years compared to the second year. Different sowing dates had a significant impact on plant height. Normal sowing resulted in taller plants while dwarf plants were observed in late sown seeds. Regarding priming treatments, AsA primed seeds exhibited taller plants under both sowing conditions which was statistically at par with those hydro-primed during both seasons. Unprimed (control) seeds resulted in dwarf plants during both sowing conditions. The interactive effect among priming treatments and sowing date were insignificant during both years.

Data analysis showed that priming treatments and sowing date significantly influenced grains per spike (Table 3). During both years varying climatic conditions exhibited significant impact as grains per spike were maximum during the first year over the second year. Regarding sowing dates, the normal sowing date resulted in maximum grains per spike than the late sowing date. Different priming treatments significantly influenced grains per spike and maximum grains per spike were noted in AsA primed seeds which were statistically the same with hydro-primed seeds. Unprimed (control) seeds have minimum grains per spike. Priming treatments and sowing dates interaction were found insignificant. A similar trend was observed during the second year as described previously.

Mean data exhibited that priming treatments and sowing dates significantly influenced 1000-grain weight during both years (Table 3). During the first year, 1000-grain weight was maximum than the second year. Interaction among priming treatments and sowing date was found significant during the first year (Figure 8) while during the second year it was insignificant. Maximum 1000-grain weight was achieved when seeds were primed with AsA which was statistically equivalence with hydro-primed seeds under normal sowing conditions (Figure 8). While these two priming treatments showed significant differences among each other under late sown conditions. Unprimed (control) seeds have the lowest 1000-grain weight under late sown conditions. Results of the main effects were differed significantly as the higher 1000-grain weight was achieved in AsA priming as compared to unprimed (control) seeds during the second year. Different sowing dates adversely affected the trait as early sown seeds resulted in higher 1000-grain weight over late sown seeds.

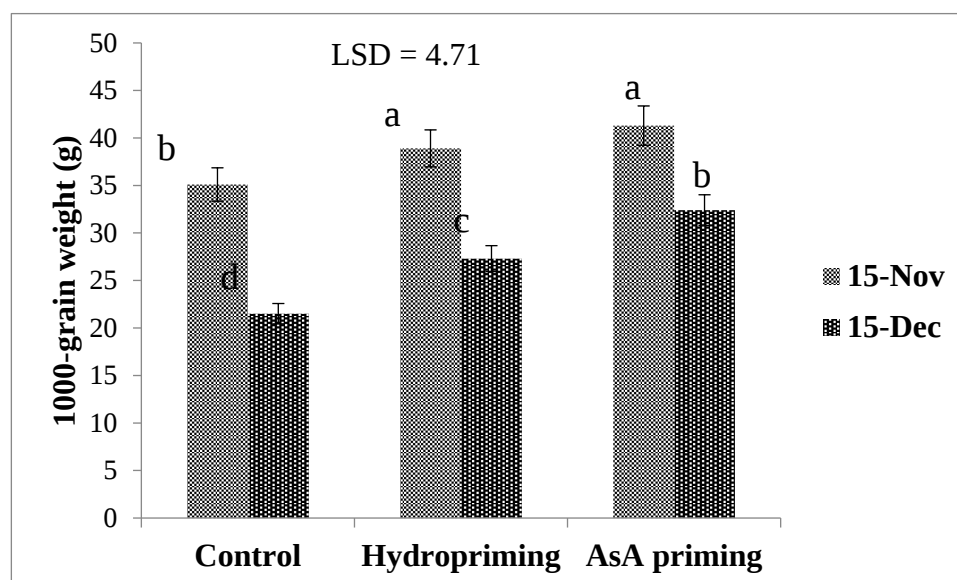


Figure 8. Interaction between priming techniques and sowing dates for 1000-grain weight during 2017. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

Grain yield was significantly influenced by priming treatments and sowing dates during both years (Table 3). However, their interaction was also found significant during the first year and insignificant during the second year (Figure 9). Data regarding grain yield showed that differences along growing years have a great impact on grain yield. Data of both years revealed that during the first-year grain yield was higher as compared to the second year (Table 3). Normal sowing gave higher grain yield as compared to late sowing during the first year. Regarding priming treatments, AsA primed seeds gave higher grain yield as compared to other treatments. While unprimed (control) seeds gave lower grain yield. AsA primed seeds resulted in higher grain yield under normal sowing conditions which was followed by hydro-primed seeds while unprimed (control) resulted in lower grain yield.

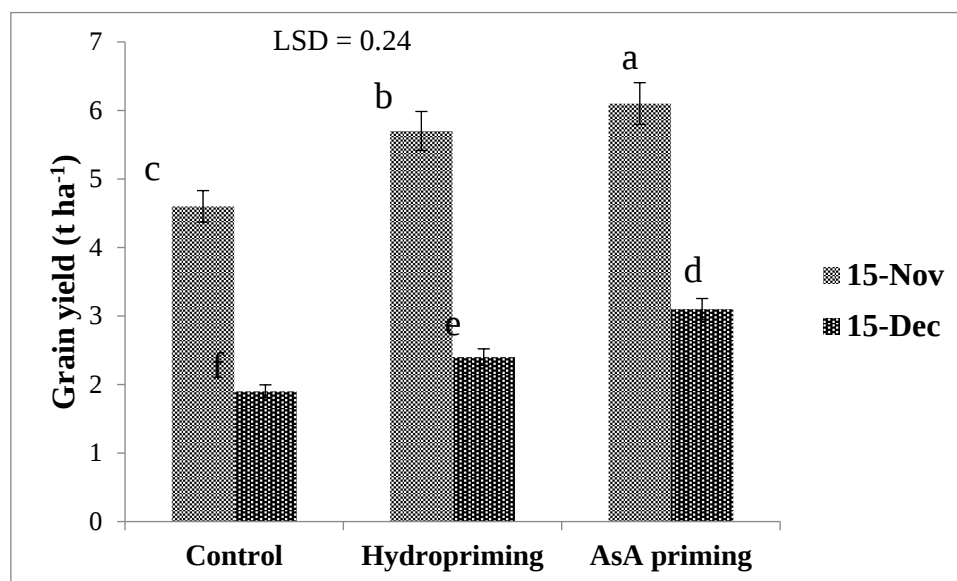


Figure 9. Interaction between priming techniques and sowing dates for grain yield during 2016. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

Data collected for biological yield exhibited that priming treatments and sowing dates greatly influenced biological yield during both years (Table 3). The interactive effect was also significant only during the second year. The more biological yield was observed during the first growing season as compared to the second growing season (Table 3). Normal sowing performed better and gave more biological yield than the late sowing. Priming treatments positively enhanced biological yield as the more biological yield was noted when seeds were primed with AsA while lower biological yield was noted in unprimed (control) seeds. Interaction effects exhibited that more biological yield was recorded from hydro-primed seeds which were at par statistically with AsA primed seeds whereas lower biological yield was recorded from unprimed (control) seeds (Figure 10).

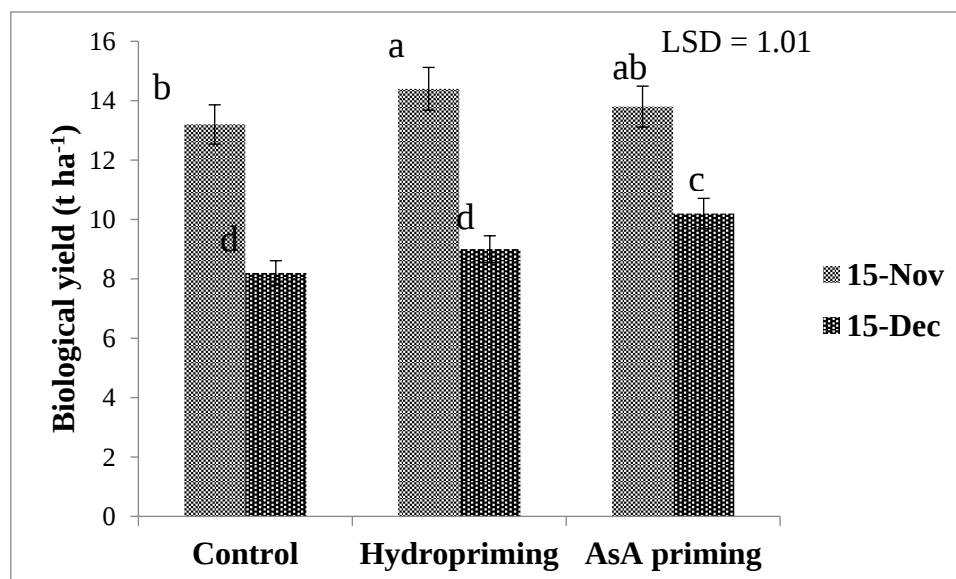


Figure 10. Interaction between priming techniques and sowing dates for biological yield during 2017. Means not sharing same letter (s) differ significantly at $p \leq 0.05$.

4. Discussion

Recently, the application of growth and yield promoters has received wide attention from plant physiologists [23,24]. Ascorbic acid can improve plant vigor and yield traits by changing plant physiological structure, enhancing nutrient acquisition and improving plant ability to cope with stress. One serious limitation for wheat yield is late sowing. It is demonstrated to be a foremost contributor to reduced wheat production, particularly in the Asian subcontinent, comprising Pakistan. Many scientists have reported the early existence of phenological periods and the decline in grain filling period, leading to the eventual decrease in the biological and economic yield of wheat due to late sowing [23]. High temperature is reported to be related to dry accumulation of wheat varieties and reduction of grain number [24]. At late sowing, high-temperature stress had a negative effect on the yield contribution of wheat tillering, anthesis, and grain filling stage. The decrease in biological and grain yield of wheat under the late sowing condition was recorded in this study.

In the current study, ascorbic acid priming was associated with higher growth and yield traits in normal and late-sown wheat (Table 1). It is clear from the results of the present study in terms of germination traits exhibited that altering sowing dates can significantly affect the mean emergence time (days), the time taken for 50% emergence (days), coefficient of uniformity emergence (CUE), and the final germination percentage (Table 1). All priming treatments enhanced the germination traits of wheat (Table 1). Higher germination traits (time to start emergence, time to 50% emergence, mean emergence time, CUE and final emergence) in wheat may be associated with better imbibition process during which seed coat breakage causes activation of enzyme activity due to seed priming [25]. Basra et al. [26] reported that the early emergence in primed seeds may also be owed to the quicker production of germination metabolites. Our study is consistent with many other studies Zheng et al. [27]; Khan et al. [1] reported the effect of seed priming on improving the emergence rate and seedling vigor. For instance, Dolatabadian and Modarressanavy [28] reported that wheat seed primed with ascorbic acid significantly increased emergence and seedling viability. Priming encourages a series of biochemical changes in seeds to initiate the pre-germination process for example inhibitors hydrolysis or metabolism, imbibition, and activation of enzymes [29]. Thus, priming seeds usually rapidly absorb and restores seed metabolism, leading to faster germination rates and maximum germination percentage [30].

During both years late sown wheat resulted in lower emergence (Table 1). The noteworthy effect of different growing seasons in this study indicated that climate change (below or above the optimal

temperature) had a prominent impact on the emergence rate and growth of wheat [31]. The decrease in emergence rate might be due to dominant weather at that time as Feng et al. [32] revealed that crops planted late were more susceptible to frost or cold stress in the early stage. Climate data (Supplementary data) depicted that the temperature in the late sowing period is below the optimum temperature. During both years, variation between December's temperatures exhibited. Therefore, it ensures a lower number of plants per unit area [33]. The results of stand establishment traits (Table 1) indicated that the total plant population was decreased. These results are consistent with the opinions of other scientists, they believe that changing the sowing date had affected stand establishment traits which impose low-temperature stress during germination and shorten the tillering period for wheat crop [34,35].

The chlorophyll content is an effective index for plants to convert solar radiation into chemical energy. The photosynthetic efficiency of plants depends on chlorophyll (a and b), which plays an important role in the light reaction of photosynthesis [36]. Farooq et al. [35] reported that late sowing led to a decrease in chlorophyll content in two varieties of barley, which may be due to the lower temperature in the early stage of vegetative growth. It has been reported that low temperature leads to decreased biosynthesis of chlorophyll content due to inhibition of chlorophyll synthesis gene expression [37]. Likewise, Ahmed and Fayyazi-ul-hassan [38] described a reduction in chlorophyll content in wheat during the late sowing. However, in the present study, seed priming improved the chlorophyll contents in both barley varieties under normal as well as late sown conditions (Table 2). This may be due to the defensive effect of seed priming through enhancing osmolytes accumulation and antioxidant activity under stress. Exogenous application of SA, AsA, and H_2O_2 augmented photosynthetic pigment in wheat [39]. According to the report and results of this study, the increased effect of seed priming strategies on photosynthetic pigment may be due to its enhanced physiological function during the normal and late sowing period.

The enzymatic antioxidant system is very helpful to overcome the adverse impacts of ROS and helps plants adapt to their surroundings. Antioxidants are normally produced in plants to isolate the activated oxygenated species of chloroplasts, mitochondria, and peroxisomes. Different sowing dates had significant effects on antioxidant activities such as superoxide dismutase (catalyzed O_2 to H_2O_2 mutation), catalase and peroxidase (scavenging H_2O_2) (Table 2). If ROS scavenging systems are insufficient, plants may suffer oxidative damage [40]. Reactive oxygen species in plants are cleared by a variety of scavenging antioxidants, which are most effective against oxidative damage [41]. In this study, AsA priming led to the increase of SOD, POD and CAT accumulation in plant tissues. The activity of CAT, SOD and POD is higher, while the activity of SOD/[CAT+POD] is low due to AsA priming, which may be due to the development of an efficient scavenging system, which generates H_2O_2 through scavenging superoxide free radicals and converts H_2O_2 to O_2 , so as to protect the membranes from freezing damage. Khan et al. [42] and Appu and Muthukrishnan [43] further confirmed our results and reported that exogenous application of AsA could improve antioxidant enzyme activity.

A higher tiller number may be the result of a higher final germination percentage (Table 3). Late sowing conditions imposed shorter life duration of plants and also cause germination loss owed to the inappropriate environmental conditions dominant during late sowing [35,44]. It increases the risk of heat-induced loss at the reproductive stage [45]. Vegetative growth negatively affected as fewer tillers per unit area, lower plant height, and lower yield is reported in our results (Table 3). Fewer tiller numbers led to lower productivity and profits. If the current farming system continues without any modifications, it will result in significant losses of vegetative and production-related traits [46]. Akhtar et al. [47] defined the lower limit of tillers generation, he indicated that the tillering stage was shortened due to unfavorable temperatures (January) and frost damage was encouraged that led to poor tiller development. The wheat sown on 10 December showed poor performance in growth and development which cause lower biomass and yield due to slow vegetative development. This was due to the extreme temperature at that time, which led to the deterioration of the quantity and

quality of crops [48,49]. These studies indicate that the authors' findings are consistent with those reported in the current work. The priming with AsA improved the adverse impacts of late sowing conditions and made the plants adapt to the harsh crop environment. The results of our study showed that the maximum tiller number could be observed in AsA primed seeds under both sowing dates whereas the more obvious effect was noted under late sown conditions (Table 3). AsA treated plants exhibited an alternate process to balance GSH/GSSG cycles with different stress response genes and transcription factors involved in upregulation. It helps plants acclimatize to the environment [50]. The increase in the previous results may be due to the increased activity of defense molecules that terminate oxygenated species and inhibit cell organelles from ROS damage [51,52]. Thus, better cell division and elongation processes control the yield of ascorbic acid-treated plants [1].

Wheat plant height is the synergistic effect of dominant climatic conditions, crop genetic potential, and growth resource availability. With significant differences in growing seasons, climatic conditions are key to determine plant height. Late sown conditions resulted in dwarf plants which might be due to low temperature occurrences and plants stopping their growth from January to February. In this study, enhanced growth by seed priming might be due to early initiation and better stress tolerance by increasing the accumulation of osmotic enzymes and antioxidant activity under stress conditions [53]. Also, osmopriming enhanced gene expression and transcription factors of isoenzymes, antioxidants, carbohydrate and nitrogen metabolism, cell development, and response to oxidative stress under stress conditions [54]. AsA priming promotes plant growth by promoting root growth, which helps in water and nutrient uptake, and the synthesis of growth-promoting hormones such as auxin, gibberellin, and cytokinin, while reducing ethylene production [55]. Many researchers have assured that pretreatment with H₂O₂ or AsA improves the retention of stay green qualities, ultimately leading to more leaf areas capturing sunlight [56]. The result proves this truth; AsA promotes plant growth to adapt to any harsh conditions [57]. The results were consistent with the results reported by Gao et al. [58] that the pretreatment effect of H₂O₂ or AsA under high-temperature stress was better than that of the control group.

Grain number per spike is a direct factor determining yield. The higher the number of grains per spike, the greater will be the final yield. However, 1000-grain weight is one of the key factors that restrict the yield potential of varieties. The 1000-seed weight is greatly affected by seed size. In this study, delayed sowing of wheat minimized the number and weight of grains per spike (Table 3). Early studies have shown that late sowing crops have to face rising temperatures during the reproductive phase, thus increasing ROS production. Similar studies have reported the effects of post-flowering stress on assimilating translocation, grain-filling, and grain size [59]. The decrease in yield contributing traits might be related to the number of flowers per plant, pollen grains, pollen viable flower buds and higher abortion rate [60]. AsA priming plays an important role in increasing spike length, spike grain number, 1000-grain weight and other yield contribution parameters (Table 3). Compared with other treatments, ascorbic acid priming had the greatest effect on spike length, spike grain number and 1000-grain weight. During the grain-filling period, AsA priming significantly enhanced 1000-grain weight gain, grain yield and biological yield due to the "stay green phenomenon". AsA priming might be related to grain filling photoassimilates translocation that eventually increasing grain weight. Khan et al. [1] reported similar findings.

Final grain yield is the product of the summation effect of various growth and yield contributing traits affected by various agronomic measures under specific ecological conditions. In this study, late sowing resulted in yield decline during both years (Table 3). The decrease in late planting yield could be attributed to the decrease in grain number and size in this study. Late sowing along with low temperature decreased net photosynthesis and increased seed abortion. Berzsenyi and Lap [61] and Namakka et al. [62] also supported these results, reporting that late planting is usually accompanied by an increase in growing season temperatures, which accelerates crop development and reduces cumulative solar radiation, resulting in reduced biomass yield, grain set, and grain production. Also, another important finding of this study was that the priming treatment (AsA priming) increased grain

production; nevertheless, AsA priming resulted in more grain and biological yield in normal and late-sown wheat (Table 3). Higher plant grain yield was correlated with AsA priming due to maximum spike length and number of spikelets. Likewise, an increase in grain yield from AsA primed seeds is due to the improvement of yield contributing factors, i.e., grain number per spike, number of spikelets per spike, grain weight, etc. Ascorbic acid is a vitamin that is rich in important phytohormones and mineral nutrients and plays an important role in improving wheat yield. Also, maximum grain yield was due to membrane stabilization and increased antioxidant activity induced by ascorbic acid which assisted wheat crop to maintain normal photosynthesis at low temperatures, which mainly occurred in the late sowing period. Previously, Yasmeen et al. [63] also described that due to the "stay green phenomenon", LAD was enhanced under MLE application and AsA priming, and the grain filling period was prolonged, resulting in a 10% increase in wheat yield at the late sowing stage. Our findings are also consistent with those of Ahmad et al. [64] and Khan et al. [1], who also reported that AsA priming helped to increase grain production under later sowing conditions.

Biological yield is the mixture of seed yield and straw yield. It is a function of crop genetic characteristics, soil nutrient status and ecological pattern around crops. In the current study, the decrease in biological yield caused by late sowing maybe because of the decrease in TDM and CGR and LAI (Table 3). Late sowing led to a decrease in plant growth and biological yield by exposing plants to cold stress [65]. Also, the decrease in biological yield in this study may be due to the decrease emergence rate and plant growth caused by low temperature in the early season, because of biological yield directly proportional to the number of plants and leaves per unit area as well as the growth rate of leaves. Our results are consistent with Farooq et al. [65] who reported that biological yield was reduced due to exposing to cold temperature. However, in the current study, ascorbic acid-priming was able to increase biological yield at the optimum sowing time and late sowing time. This may be because under normal and stress conditions, the early seedling vigor is enhanced and the plant's protection against environmental stress is also enhanced, thus improving the growth and leaf area of the plant [66]. Also, seed priming has been observed to improve photosynthetic pigments, which may lead to increased photosynthesis, thus increasing TDM yield, biological yield, and grain yield [67].

5. Conclusions

In summary, late sowing had inhibitory effects on all emergence, biochemical, yield and yield-related traits in wheat, however, seed priming with AsA (50 mg L⁻¹) was more effective in promoting germination characteristics, chlorophyll content, and antioxidant capacity under varied sowing conditions than varied sowing conditions compared to non-primed seeds. This allows the plant to perform better in the late sowing period. Late sowing crops showed acclimatization to the stress of late sowing when primed with AsA. The decline in oxidative damage may enable wheat to maintain a high content of chlorophyll, thus improving wheat under the late sowing condition. Our results show the beneficial effects of AsA priming on key crops such as wheat, and study of this experimental approach from the perspective of stand establishment, biochemical, yield and yield-related traits. Therefore, under the condition of late sowing, the acquired thermo-tolerance can be achieved through AsA priming.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/9/11/757/s1>, Figure S1: Climatic data during crop season 2016–2017, Table S1: Physiochemical analysis of soil during 2016–2017.

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