The role of wild rice (Oryza rufipogon) awns in seed dispersal

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9

10   Running head title

11   Role of awns in wild rice, *Oryza rufipogon*

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22

1 **Abstract**

2 Cultivated rice (*Oryza sativa* L.) was domesticated from the Asian wild species *O. rufipogon*.

3 Compared with cultivated rice, wild rice has spikelets/seeds with long barbed awns. In order to

4 evaluate the role of awns in wild rice, four seed groups with different awn lengths (full, ½, ¼,

5 and no awns) were prepared, and the following seed dispersal movements were investigated

6 under simulated natural conditions as observed in the tropical Asian habitat: (1) Seed

7 detachment from the parent plant. (2) Falling angle of mature seed. (3) Ability to slip into small

8 spaces. (4) Horizontal movement on the ground. (5) Horizontal movement in water. As a result,

9 awns were found to enhance the detachment of matured seeds from the panicles in the initial

10 seed dispersal step. They regulated vertical orientation in the air, and the vertical form may

11 enable seeds to squeeze to the ground. The awned seeds also showed advantages in horizontal

12 movements on the ground and in the water. In most of the experiments, seeds with full awns

13 showed the best performance for seed dispersal, suggesting that wild rice keeps long awns to

14 survive under natural conditions. Since seed awning is dominantly controlled by wild functional

15 alleles at several loci, wild rice may be able to prevent drastic reduction of awn length.

16

17 **Introduction**

18 Cultivated rice (*Oryza sativa* L.) was domesticated from the Asian wild species *O. rufipogon*

19 Griff. (Oka, 1988). *O. rufipogon* grows mainly in swamps, ditches, canals, and ponds under

20 natural tropical conditions (Figs. 1 and S1). Compared with cultivated rice, wild rice has

21 spikelets with long barbed awns (bristle-like organs at the tips of the lemmas) on panicles and

22 the matured grains with hulls (hereafter seeds) shed by themselves (Fig. 2). Many studies have

been carried out to clarify the genetic regulation of seed awning using interspecific populations between wild and cultivated rice species, and three major loci, *An-1*, *LABA1*, and *RAE2*, were identified (Luo *et al.*, 2013; Hua *et al.*, 2015; Bessho-Uehara *et al.*, 2016). At each locus, the wild functional allele induces awn formation. In addition, wild rice has some other minor genes for seed awning, and an awnless phenotype is not produced by a single mutation in wild rice (Ikemoto *et al.*, 2017; Amarasinghe *et al.*, 2020). These suggest that wild rice has a mechanism to prevent awn length reduction by the multiple genetic controls.

In wild plants, long awns assist seed dispersal. Chambers and MacMahon (1994) defined two phases of seed dispersal: seed movement from the parent plant to a surface (Phase I) and subsequent horizontal or vertical movement (Phase II). In wild wheat, awns propel the seeds on the ground and silica hairs covering the awns push the seeds into the soil during the daily humidity cycle (Elbaum *et al.*, 2007). Similarly, awns with micro-barbs have been found to promote seed dispersal and burial in wild foxtail grass (Kulic *et al.*, 2009; Wolgemuth, 2009). In wild rice, mature seeds have strong dormancy enabling survival in the tropical dry season, and they germinate the following rainy season (Oka, 1988). The efficient dispersal of the seeds directly contributes to the expansion of distribution area in the next generation. Wild rice has seeds with long barbed awns, however, we do not well-understand their roles on seed dispersal under natural conditions.

In the present study, we prepared seeds with different awn lengths and conducted seed dispersal experiments under simulated natural conditions as observed in the tropical Asian habitat. In Phase I of seed dispersal, seed detachment from the parent plant and seed falling angle in the air were examined. In Phase II, seed slipping ability into small spaces and

1 horizontal movement on the ground and in water were investigated. In each condition, seed

2 dispersal performance was quantified to clarify the roles of awns in wild rice.

3

4 **Materials and methods**

5 *Plant materials*

6 A long-awned accession of *O. rufipogon* W630, provided by the National Institute of Genetics,

7 Japan, was used in this study. This accession was previously examined in domestication and

8 genetic studies (Ishii *et al*., 2013; Ikemoto *et al*., 2017).

9 *Seed dispersal experiments*

10 In tropical Asia, *O. rufipogon* grows mainly in wet areas, and the matured seeds naturally shed

11 on the ground or into shallow water. To evaluate the roles of wild rice awns in seed dispersal in

12 Phases I and II, the following five conditions were set for the flowering spikelets and mature

13 seeds with different awn lengths.

14 (1) Seed detachment from the parent plant (Phase I)

15 The first seed movement from the parent plant (Phase I) is a detachment of matured seed from

16 the panicle. In wild rice, panicle flowering begins with spikelets in the upper part of the panicle,

17 and is complete in about 10 days. And it takes about two weeks for each seed to mature. To

18 evaluate the role of awns in seed detachment from the parent plant, 16 plants of *O. rufipogon*

19 W630 were grown in pots under natural conditions. From August 21 to 28, two panicles that had

20 started flowering were selected from each plant, and the awns of the spikelets were cut to give

21 four kinds of awn lengths; full, ½, ¼, and no awns (Fig. 3). In each awn treatment, eight

22 panicles (two panicles each from four plants) were prepared, and the spikelets that flowered in

4

1 the first 7 days were painted different colors to mark the flowering dates. Then, the days to

2 shedding from flowering were examined for the matured seeds by daily observation. The

3 average value of two panicles was taken as the days to seed shedding for each plant, and the

4 overall averages were compared among the four groups with different awn lengths.

5 (2) Falling angle of mature seed on muddy ground (Phase I)

6 Wild rice plants grow in swampy areas and panicles emerge vertically at heading stage (Fig. 1).

7 Their mature seeds sometimes fall on muddy ground (Fig. S1A, B). In rice, embryos develop in

8 the glumes on the side opposite the awns. Therefore, angles of inclination to the ground are

9 important for seeds to self-plant the embryo side down on muddy surface. To examine whether

10 the awns are associated with the falling angles of mature seeds, long awns (about 10 cm) of

11 mature seeds were cut, and four seed groups with different awn lengths were prepared: full

12 (about 10 cm), ½ (about 5 cm), ¼ (about 2.5 cm), and no (0 cm) awns (Fig. 4). The seeds were

13 dropped in a vertical position from 1 m above the muddy ground (Fig. S2A), and the angles of

14 inclination to the ground were scored on a scale of 0 to 5: 0, 0–15°; 1, 15–30°; 2, 30–45°; 3, 45–

15 60°; 4, 60–75°; 5, 75–90° (Fig. S2B, C). The average score of ten seeds was calculated for each

16 awn treatment with ten replications.

17 (3) Ability to slip into small spaces covered by plants (Phase II)

18 Wild rice is often found in weedy wetland areas. The shed seeds must pass through the surface

19 covered with stems and leaves of other plants (Fig. S1C, D). This corresponds to the vertical

20 movement of seed dispersal in Phase II. To inspect the ability of awned seeds to slip into small

21 spaces, an apparatus with four layers (at either 2- or 4-cm intervals) of plastic mesh was

22 prepared (Fig. S3). The mesh size was 4 mm, which exceeded the seed width (about 2.5 mm)

5

but was less than the seed length (about 7.5 mm). Seeds with four variations of awns were dropped in a vertical position from 1 m above the apparatus. Slipping ability was scored based on the number of mesh sheets passed through, namely, 0 (stopped on the first mesh) to 4 (passed all four mesh sheets). The seed trial (ten seeds with ten replications for each awn group) was the same as in the above falling angle experiment.

(4) Horizontal movement on the ground (Phase II)

A fourth experiment was conducted to examine the horizontal movement of awned seeds on the ground. In the dry season, wild rice seeds are often found half-buried in the ground (Fig. S1E, F). Wild rice has many small upward silica barbs on the awn surface, and these barbs direct the direction and movement of the seeds (Hua *et al*., 2015). The horizontal movement of awned seeds was examined using a reciprocal shaker with a swing of 5 cm (SR-1; TAITEC Co., Japan). Because awnless seeds move easily by rolling on a flat surface, a V-shaped plastic rod (2 × 2 cm at the edge, 80 cm in length) covered with filter papers (3MM; Whatman, UK) was set on the shaker (Fig. S4). First, seed movement was checked at three shaking speeds (150, 175, and 200 rpm) using 100 seeds each for the four awn treatments: full, ½, ¼, and no awns. Then, at two appropriate speeds (175 and 200 rpm), the average velocity (in 80 cm) of ten moving seeds was calculated for each awn group, with ten replications (Supplementary Movie S1).

(5) Horizontal movement in water (Phase II)

The mature seeds of wild rice growing near a small river or ditch usually drop into the water (Fig. S1G, H). Seeds are also covered with water in flooded areas. These seeds may spread horizontally with water movement. Therefore, an experiment on seed movement in water was designed using a circulating water apparatus (Nagashi-Somen; Kano Co., Japan) (Fig. S5). For

1   two water speeds (5 and 15 cm/s), the velocity of the seeds with four length variations of awns

2   (full, ½, ¼, and no awns) was estimated based on the distance moved in 60 s (Supplementary

3   Movie S2). The average velocity of five seeds was first calculated for each awn treatment, and

4   the overall averages were compared with four replications.

5   *Statistical analysis*

6   The significance of the overall averages among the four groups with different awn lengths was

7   tested using the Tukey−Kramer test, with $p < 0.05$ indicating statistical significance.

8

9   **Results**

10   *Seed dispersal experiments*

11   (1) Seed detachment from the parent plant

12   A total of 1,799 spikelets (or fertile seeds) from 32 panicles were observed (Table S1), and the

13   average numbers of spikelets in a panicle for the full, ½, ¼, and no-awn treatments were 55.6,

14   62.1, 53.8, and 53.4, respectively. The plant group with no awns had the longest average days to

15   seed shedding (15.41 days), followed by that with ¼ awns (14.72 days). Similar values were

16   observed for the groups with half (14.46 days) and full (14.49 days) awns. The days to seed

17   shedding were significantly longer ($p < 0.05$, Tukey−Kramer test) only for the group with no

18   awns (Fig. 5A).

19   (2) Falling angle of mature seed on muddy ground

20   Supplementary Table S2 summarizes the scores of the experiment on seed falling angle. The

21   scores for the full, ½, ¼, and no-awn groups were 4.51, 4.08, 2.94, and 0.40, respectively. The

22   falling angle scores of the full and half awn groups were similar, while the ¼ and no-awn

groups each differed significantly from the others (Fig. 5B).

(3) Ability to slip into small spaces

Supplementary Table S3A shows the average scores of ten seeds for the four awn groups. In the both experiments at 2- and 4-cm intervals, the scores of the full and half awn groups exceeded 3.16, showing that many seeds passed all mesh sheets. For the seeds with ¼ awns, the overall average scores were 2.73 and 2.09 at 2- and 4-cm intervals, respectively. On the other hand, most awnless seeds could not pass the first mesh sheet at both intervals, the scores for no awns were less than 0.4. At both intervals, the full and half awn groups did not differ significantly, but detected the ¼ and no-awn groups differed significantly ($p < 0.05$, Tukey−Kramer test; Fig. 5C, D). The t-tests were additionally carried out between the scores at two intervals in each awn group. The significant result ($p < 0.01$) was detected only for the seeds with ¼ awn length (Supplementary Table S3B).

(4) Horizontal movement on the V-shaped rod

At first, seed movement was compared at three shaking speeds (Supplementary Table S4A). We observed almost half of the awnless seeds did not move at 150 rpm. Therefore, the average velocity (for 80 cm) of the moving seeds at 175 and 200 rpm was further calculated for each awn group (Supplementary Table S4B). Compared to the ¼ and awnless seeds, significantly higher seeds (about 14 cm/s at 175 rpm and about 20 cm/s at 200 rpm; $p < 0.05$, Tukey−Kramer test) were recorded for the seeds with full and half awns at both shaking speeds (Fig. 6A, B).

(5) Horizontal movement in water

The averages for the four groups with different awn lengths are shown in Supplementary Table S5. At a water speed of 5 cm/s, only full awn seeds moved smoothly, whereas all seeds moved

1   at a speed of 15 cm/s. The average seed velocities were significantly faster ($p < 0.05$, Tukey–

2   Kramer test) in the full-awn group at a water speed of 5 cm/s and the full and half awn groups at

3   a water speed of 15 cm/s (Fig. 5C, D).

4

5   **Discussion**

6   Seed shattering is an important characteristic enabling wild rice to survive under natural

7   conditions. Wild rice starts to form an abscission layer between the grain and pedicel in the

8   early stage of spikelet development (Li *et al.*, 2006). Once the abscission layer formation is

9   completed, the matured seeds are ready to detach from the panicles. In this study, the average

10   days to seed shedding were 14.49 and 14.46 for full and half awn plants, respectively. These

11   were about 1 day shorter than that for no-awn plants (15.41 days). Although, the plants with ¼

12   awns had a slightly higher average (14.72 days), the difference in days to shedding differed

13   significantly between the awned and awnless groups (Fig. 5A). These results demonstrate that

14   awns enhance the detachment of matured seeds from the panicles in the initial seed dispersal

15   step of Phase I.

16   Detached seeds fall through the air, and the falling form is critical for the first contact

17   with the ground. Therefore, we examined the falling angle of the seeds, and prepared four seed

18   groups with different awn lengths (Fig. 4). According to the angle scores, the seeds with full and

19   half awns fell almost vertically, those with ¼ awns dropped with slight inclination, and most

20   awnless seeds rotated randomly as they fell (Fig. 5B). This suggests that awns regulate vertical

21   orientation via air resistance. The falling angles of the seeds directly affect self-planting in

22   muddy ground. Namely, awned seeds can push the embryo side down into the mud. The vertical

9

form may enable seeds to squeeze to the surface when the ground is covered with other plants.

In the seed slipping experiments, the awned seeds passed many mesh layers, while most awnless seeds were blocked by the first sheet (Fig. 5C, D). Interestingly, for sheet intervals wider than the seed length (for seeds with ¼ awns at 4-cm interval), the slipping scores decreased significantly (Supplementary Table S3B). These results indicate that long awns facilitate the first vertical penetration to the ground.

After seed dispersal in Phase I, mature seeds must undergo subsequent horizontal movement. Because wild rice grows in wet areas, seeds fall to the ground or into water. In the dry season, water is drained and the ground surface becomes hard. Therefore, we examined the horizontal movement of seeds on hard ground and in water. In the shaker experiments, awned seeds moved more than awnless seeds on the rod (Supplementary Table S4A). Although small hairs were observed on the seed surface (Fig. 2C), half of the awnless seeds could not move at a shaking speed of 150 rpm. At higher speeds of 175 and 200 rpm, most of the seeds moved, suggesting that the anisotropic friction produced by upward awn barbs and seed hairs allowed the seeds to move. During the forward movement of the shaker, the upward barbs and hairs hooked the surface, and they were released and slipped during backward movement. The strength of the friction also reflected the seed velocity. Therefore, significantly faster speeds were observed for the seeds with more barbs (i.e., longer awns; Fig. 6A, B). In the water flow experiments, the seeds with long awns also tended to move faster (Fig. 6C, D). Interestingly, the seeds moved awn-forward, as though the seeds were pulled by the long awns (Supplementary Movie S2). Awns probably have a lower specific gravity than the seed bodies in the water. In both experiments, the awned seeds showed advantages in horizontal movements on the ground

and in the water, implying that the awns are also necessary for seed dispersal in Phase II.

This study quantified the roles of awns experimentally in Phases I and II of seed dispersal (Chambers and MacMahon, 1994). In most of the experiments under simulated natural conditions, seeds with full awns showed the best performance for seed dispersal, suggesting that wild rice keeps long awns to survive under natural conditions. Similarly, seeds with half awns showed better contribution for seed dispersal. In wild rice, seed awning is dominantly controlled by wild functional alleles at several loci, and the plants with drastic reduction of awn length are hardly produced (Luo *et al*., 2013; Hua *et al*., 2015; Bessho-Uehara *et al*., 2016; Ikemoto *et al*., 2017; Amarasinghe *et al*., 2020). In other wild grass species, long awns were also reported to assist seed dispersal and burial (Garnier and Dajoz, 2001; Li *et al*., 2015; Ntakirutimana *et al*., 2019). Since these seed movements are essential for the successful seed propagation, their awn lengths may be maintained under similar genetic controls in wild rice.

This study demonstrates how barbed awns contribute to successful seed propagation in wild rice. Through the experiments under simulated natural conditions as observed in the tropical Asian habitat, long awns of wild rice were well-studied to have positive effect on seed dispersal in many phases. The present results may provide fundamental information for the ecological studies on wild propagation strategies and domestication studies on awnlessness in rice.

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6

7 **Figure legends**

8 **Fig. 1.** Wild rice grown in tropical Asia. (A) A large population near a paddy field in Phnom

9 Penh, Cambodia. (B) A small patch beside a ditch in Cantho, Vietnam.

10 **Fig. 2.** Awns of wild rice. (A) Maturing seeds on the panicles. Left: *O. sativa* cv. IR36. Right:

11 *O. rufipogon* acc. W630. Scale bar = 5 cm. (B) Micro-barbs observed on the surface of a

12 wild awn. Micrometer unit = 1 mm. (C) Upper side of a wild seed. The same magnification

13 as in (B).

14 **Fig. 3.** Panicles observed for seed detachment. Awns of spikelets were cut to give four awn

15 lengths: (A) full, (B) ½, (C) ¼, and (D) no awns.

16 **Fig. 4.** Seeds treated to give different awn lengths: full, ½, ¼, and no awns. Scale bar = 5 cm.

17 **Fig. 5.** Comparisons of the means of the groups with full, ½, ¼, and no awns. (A) Days to

18 shedding. (B) Seed falling angle. (C, D) Seed slipping scores at 2- and 4-cm intervals,

19 respectively. Means labeled with different letters differ significantly (Tukey−Kramer test, $p <$

20 0.05).

21 **Fig. 6.** Comparisons of the means of the groups with full, ½, ¼, and no awns. (A, B) Seed

22 moving speeds on the rod at shaking speeds of 175 and 200 rpm, respectively. (C, D) Seed
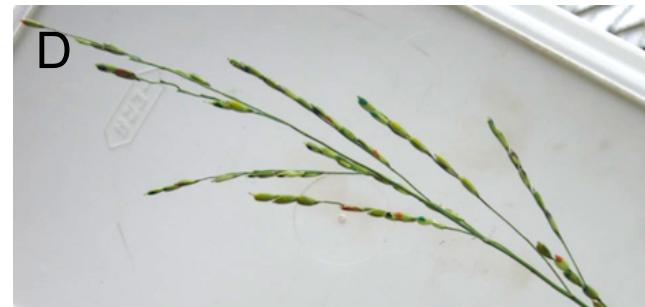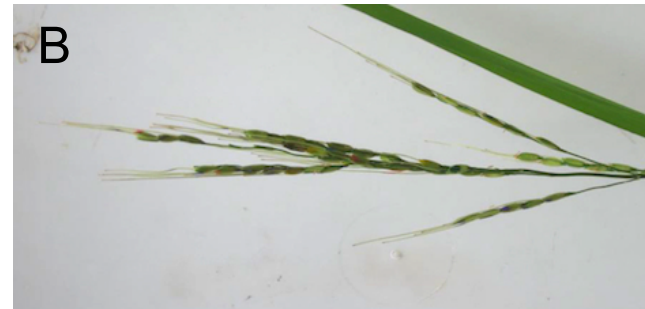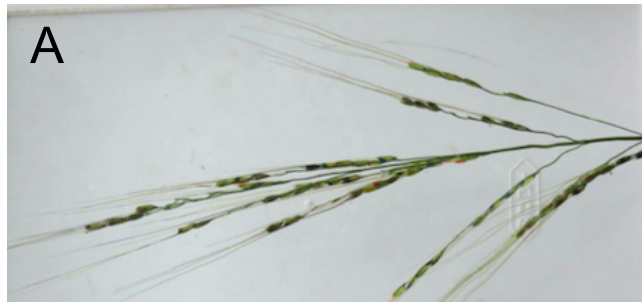
1   speeds in the water under water flow speeds of 5 and 15 cm/s, respectively. Means labeled

2   with different letters differ significantly (Tukey−Kramer test, $p < 0.05$).
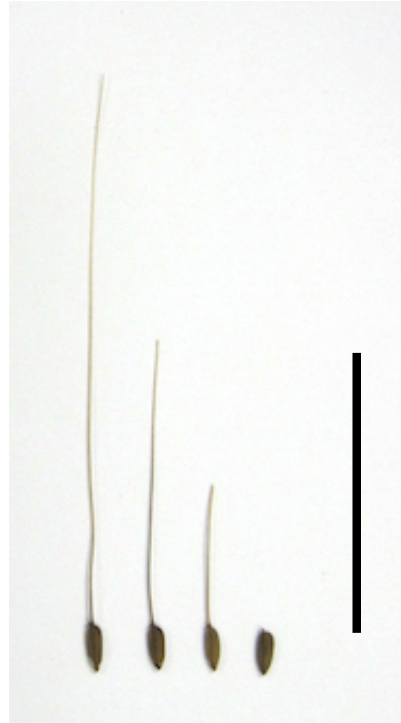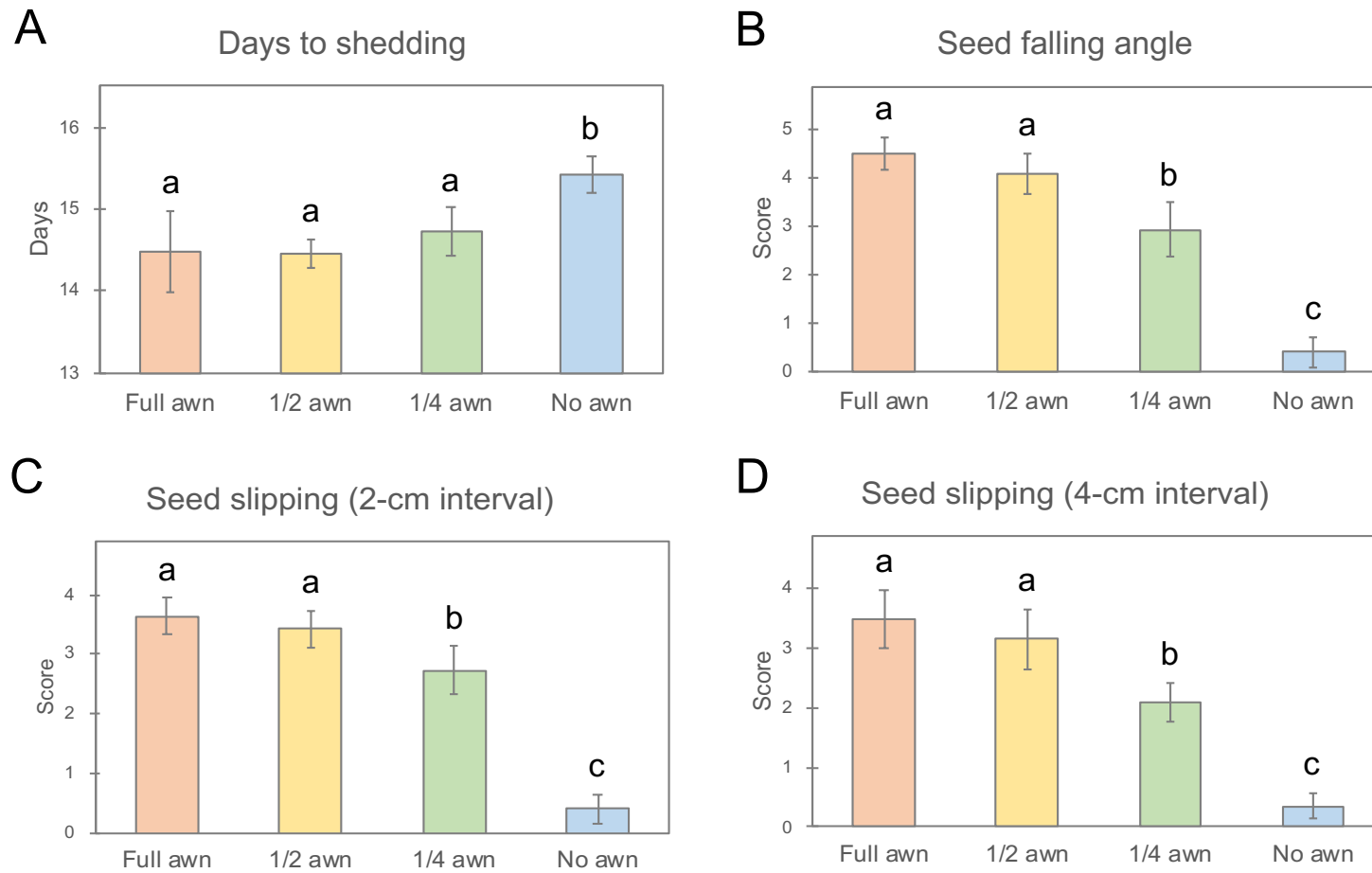
A — Days to shedding
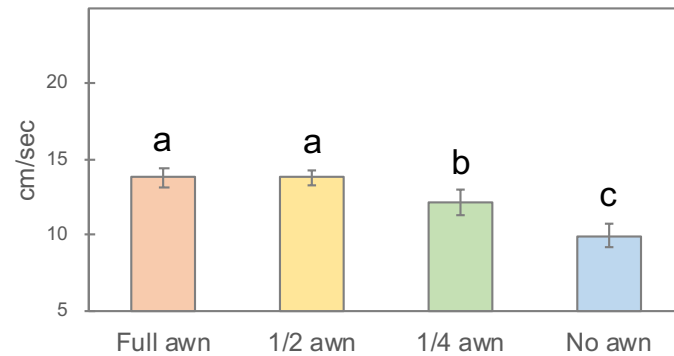B — Seed falling angle
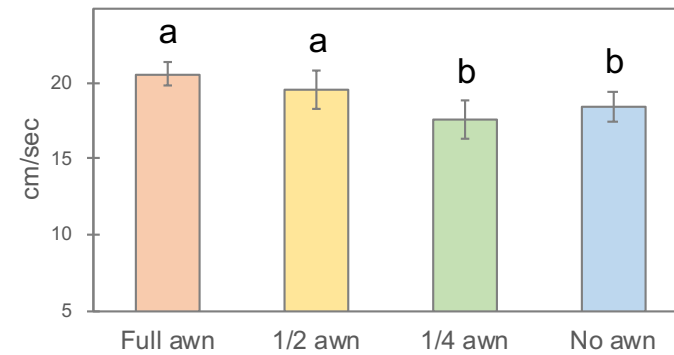C — Seed slipping (2-cm interval)
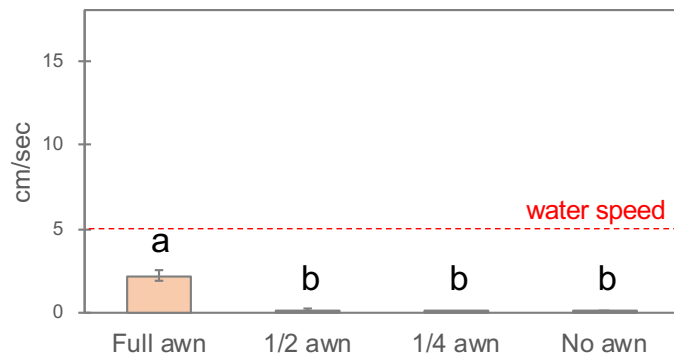D — Seed slipping (4-cm interval)

A Moving speed on the rod (175 rpm)

B Moving speed on the rod (200 rpm)

C Moving speed in the water (5 cm/sec)

D Moving speed in the water (15 cm/sec)