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Germination ecology of heteromorphic seeds of bur clover (*Medicago denticulata* willd.)

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ABSTRACT

Bur clover (*Medicago denticulata* Willd.) is a winter annual weed commonly found in wheat fields of Punjab, India. We observed it produces heteromorphic seeds varying in seed coat colour - cream and brownish-black. The information on germination ecology of *M. denticulata* seeds varying in seed coat colour is lacking. So, the present study was undertaken under laboratory conditions to study the effect of seed coat colour on germination characteristics of *M. denticulata* seeds in relation to various environmental variables. Germination of both cream and brownish-black seeds was independent of light. Cream seeds germinated in the wide temperature range of 15/5 to 30/20°C; while, brownish-black seeds germinated in a narrow temperature range of 15/5 to 25/15°C. Cream seeds were able to withstand greater salinity stress as some seeds (10%) were able to germinate under NaCl concentration of 200 mM; whereas germination of brownish-black seeds was completely inhibited at 200 mM NaCl. The NaCl concentration required for 50% inhibition of maximum germination for cream and brownish-black seeds was 100 and 70.6 mM NaCl, respectively. The osmotic potential required for 50% inhibition of the maximum germination of cream and brownish-black seeds was -0.37 and -0.32 MPa, respectively. These results indicated a greater ability of cream seeds to tolerate either salinity or osmotic stress. Both seeds germinated under acidic and alkaline pH with >40% germination in pH range 3-10. The highest emergence of cream and brownish-black seeds was recorded when seeds placed on the soil surface.

INTRODUCTION

Seed heteromorphism is an important phenomenon which enables a single plant species to produce seeds with different shape, size, colour and germination behaviour (Baskin and Baskin 2014). The acquisition of seed coat colour depends on environment, sequential developments on the maternal plants and/or may be genetically inherited (Liu *et al.* 2007). Liu and Wei (2007) observed that the rate of germination of brown seeds of *Atriplex micrantha* was significantly higher as compared to black seeds at three tested temperatures *viz*., 5/15°C, 5/25°C and 15/25°C. Environmental factors such as temperature, light, soil pH, moisture and salinity modulate the germination and seedling growth and thereby influence the emergence of weed seedlings in the field (Koger *et al.* 2004). Light has been reported to promote germination of some weed species, *viz*. toothed dock (*Rumex dentatus*) and common lambs quarters (*Chenopodium album*) (Al-Helal 1996, Dekker 2014). On the contrary, some species like *Convolvulus arvensis* and *Lathyrus aphaca* (Kumari *et al.* 2010) best germinated under darkness. Temperature is directly related to water absorption and various biochemical reactions occurring in the seeds thereby regulating the germination process. Moisture is a basic requirement for germination and lack of water may delay, reduce, or prevent seed germination (Javaid and Tanveer 2014). Burial depth has also been reported to influence the germination, dormancy, and viability of seeds by influencing the availability of moisture and light (Chauhan and Johnson 2010).

Bur clover of fabaceae family, is an annual winter weed which has invaded many states of India, *viz*. Punjab, Haryana, Jharkhand, Bihar, Madhya Pradesh and West Bengal. Among the various dicotyledonous weeds, *M. denticulata* is the major problematic weed prevalent in wheat fields of Punjab (Chhokar *et al.* 2006). We observed that *M.*

Plate 1. Heteromorphic seeds (cream and brownish-black) of *Medicago denticulata* Willd.

denticulata produces heteromorphic seeds that differ in seed coat colour- cream and brownish-black (**Plate 1**). Some information is available on the effect of environmental factors on germination ecology of *M. Polymorpha* (Wagner and Spira 1994). However, no information is available regarding dormancy and germination response of heteromorphic seeds of *M. denticulata* in relation to various environmental factors. Detailed information on environmental factors which influence the seed germination process may help to optimize better weed management decisions. So, the present study was undertaken to evaluate the effect of seed coat colour on germination characteristics of *M. denticulata* seeds in relation to various environmental variables.

MATERIALS AND METHODS

Collection of seeds

Pods of *M. denticulata* containing mature seeds were collected from Research Farm, Department of Agronomy during the months of April 2016. Seeds were removed from pods immediately before use. Two seed lots - cream and brownish-black were prepared by visual inspection of seed coat colour. Brownish-black seeds were non-dormant and germinated rapidly after 1 month of harvest; while the cream seeds were dormant. They possessed seed coat imposed dormancy and were scarified by hand with sandpaper for 1 minute for breaking the dormancy before conducting each experiment.

Germination protocol

Seed germination was tested by placing 30 uniform sized seeds of *M. denticulata* from each seed lot in 9 cm Petri dishes lined with Whatman No. 1 filter paper. For studying the effect of light, temperature, moisture stress, salinity and pH, Petri dishes were moistened with 5 ml of treatment solution and incubated at 20°C (optimal temperature) in an environmental chamber (Model MAC MSW-127, Delhi, India). For the control treatment, seeds were germinated using distilled water only.

Temperature

Seed germination was tested under five alternate day/night temperatures (12 h light/12 h dark), *viz.* 15/5, 20/10, 25/15, 30/20 and 35/25°C using three replicates.

Light

To study the effect of light on germination, Petri dishes were kept under three light regimes- continuous light (24 h), light/dark (12/12 hours) using a light intensity of 85 mmol m^{-2} s^{-1} and continuous dark (24 h) at 20°C. In the latter treatment, Petri dishes were wrapped with double layers of aluminum foil immediately after adding distilled water to completely obstruct penetration of light. The data on germination counts were recorded on 15th day after the initiation of the experiment.

Moisture

The ability of seeds to germinate under different levels of moisture stress was tested using solutions of PEG 8000 having water potentials of 0, -0.1, -0.2, -0.4, -0.6, -0.8 and -1.0 MPa (Michel and Kaufmann 1973).

Salinity

The ability of seeds to germinate under different salt stress levels was examined by using NaCl solutions of 25, 50, 75, 100, 150, 200 and 250 mM concentrations.

pH

The effect of pH on seed germination was investigated using buffered solutions with pH ranging from 3 to 10 (Chachalis and Reddy 2000). Unbuffered distilled water (pH 6.6) was used as control.

Burial depth

This experiment was conducted using 25-cm diameter plastic pots placed under field conditions during November-December 2016-17 and 2017-18. Soil filled in these pots was collected from those fields which recorded no previous incidence of this weed. Fifty seeds of both seed lots were sown on the soil surface in pots and covered to a depth of 0, 1, 2, 4, 6, 8 and 10 cm. The pot surface was kept moistened throughout the study period. The emergence was recorded over a period of one month. One set of pots was also kept in which no seeding of this weed was done to eliminate the error. This experiment was conducted using four replications each time.

Observations recorded

Germination counts were daily made for 15 days after start of the experiment. The seeds showing visible protrusion of radicle were considered as germinated. Germination count was calculated as germination (%) = [Number of seeds germinated / total number of seeds] × 100.

Speed of germination (germination index) was calculated as described by Association of Official Seed Analysts (1983). Mean germination time (MGT) was calculated as suggested by Ellis and Roberts (1981). Seedling vigour index (SVI) was calculated using the following formula given by Abdul-Baki and Anderson (1973):

Seedling vigour index I = seedling length (cm) x germination (%)

Statistical analysis

All the experiments, were conducted three times in a completely randomized design using three replicates. data were pooled and analyzed (ANOVA) using statistical analysis software version 9.2 (SAS 2009). Means were separated at $\alpha \leq 0.05$ using Fisher's Protected Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Effect of day/night temperature

Cream seeds possessed the ability to germinate in the temperature range of 15/5 to 30/20°C with the highest germination (%) at 25/15°C (**Table 1**). Maximum germination speed and minimum germination time was observed at day/night temperature of 25/15°C. Cream seeds germinated at day/night temperature of 30/20°C took one extra day for initiation of germination along with the reduced speed of germination and increased mean germination time. No germination of cream seeds was observed at day/night temperature of 35/25°C. Germination of brownish-black seeds occurred in a narrow temperature range of 15/5 to 25/15°C with maximum germination at 20/10°C; however, germination was completely inhibited at 30/20°C. Seeds exhibited maximum germination speed and minimum mean germination time at temperature 20/10°C. With an increase in temperature above 20/10°C, there was an increase in mean germination time along with a reduction in germination (%) and speed of germination. Cream seedlings grown at 25/15°C exhibited the greatest seedling vigour index indicating their higher competitiveness than seedlings grown at other temperatures. The highest vigour of brownish-black seedlings was recorded at 20/10°C temperature with a significant decrease in vigour index with further increase in temperature (**Figure 5a**). Like *M. denticulata*, many other dicotyledonous weeds such as *C. album* and *R. dentatus* have been reported to germinate under a wide range of temperature from 5-25°C (Benvenuti *et al.* 2001, Tanveer *et al.* 2009). The ability of weeds to germinate across a wide range of temperatures suggests their ability to emerge throughout the cropping season making weed management difficult. This flexible germination habit of weeds provides opportunities for weed proliferation, leading to abundant seed production. The results of present study indicate that cream seeds can germinate in a broad range of temperatures (15/5-30/20°C) whereas germination of brownish-black seeds can occur only in a narrow temperature range (15/5-20/10°C). This implies robust germination characteristics of cream seeds as compared to brownish-black seeds. It is also important to mention here that cream seeds exhibited germination only after scarification indicating seed coat-imposed dormancy and greater mechanical strength of cream seeds which could contribute to the perpetuation of seeds under adverse environmental conditions. Cream seeds may also be able to emerge in multiple flushes as and when their seed coat-imposed dormancy is relieved under natural conditions. A gradual loss of physical dormancy under natural conditions could be due to the action of soil microbes on the seed coat or abrasion of seed coat by soil particles (Zalamea *et al.* 2015).

Effect of light

Germination of both cream and brownish-black seeds did not differ significantly under light and dark conditions, which indicates that seeds of this species are non-photoblastic (**Figure 1**). However, dark

Table 1. Effect of day/night temperature regime on germination of cream and brownish-black seeds of *M. denticulata*

Temperature (°C) (12 h light/12 h dark)	Germination (%)		Time to start germination (days)		Germination speed		Mean germination time (days)	
	C	B	C	B	C	B	C	B
15/5	65.5	54.4	2.0	2.0	4.4	4.2	6.2	6.4
20/10	88.7	80.0	2.0	2.0	10.1	9.0	3.3	3.4
25/15	96.6	72.2	2.0	2.0	10.4	7.2	3.1	4.6
30/20	54.4	NG	3.0	NG	2.8	NG	6.6	NG
35/25	NG	NG	NG	NG	NG	NG	NG	NG
LSD (p=0.05)	7.67	7.52	3.4×10^{-6}	2.4×10^{-6}	0.17	0.13	0.11	0.12

C-Seeds with cream seed coat; B-Seeds with brownish-black seed coat; LSD- Least significant difference; NG-No germination grown seedlings of both were etiolated having pale yellow coloured leaves and elongated shoots. Dark grown cream and brownish black seedlings exhibited higher values of seedling vigour index I than seedlings growing under light conditions (**Figure 5b**). Light is an important ecological determinant for germination and the absence of light acts as a soil depth indicator that prevents germination of many weed species (Crisraudo *et al.* 2007). Seed germination response to light may vary considerably from species to species.

Ghadiri and Niazi (2005) reported that light effectively stimulated germination of dicotyledonous weeds - *R. dentatus* and *C. album* which indicates that both species are positively photoblastic and germination in the field will be favoured by presence of seeds at the soil surface. The results of present study indicate that unlike these winter weeds, germination of both cream and brownish-black seeds of *M. denticulata* is independent of light, so this weed can emerge not only from soil surface but also from different soil depths. Thus, as a management strategy, tillage operations that invert soil and bury seeds in deeper soil layers may not be successful in preventing the germination of this weed.

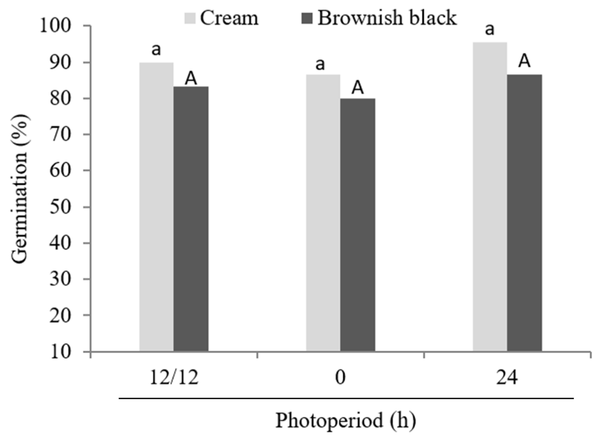


Figure 1. Effect of light on germination of cream and brownish-black seeds of *M. denticulata* Willd. under variable photoperiod. Columns followed by same lower case (cream) and upper-case letters (brownish-black) do not differ significantly at 5% level of significance

Effect of moisture stress

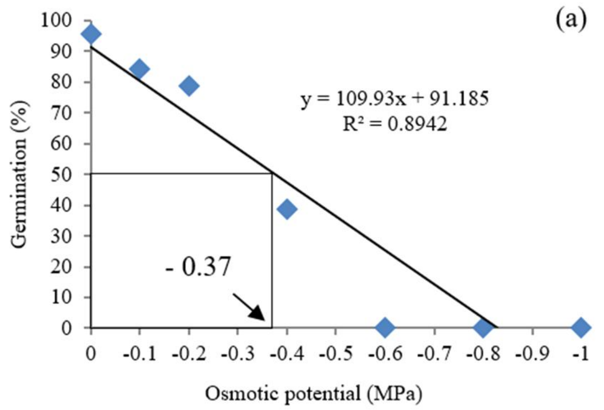
Both cream and brownish-black seeds were sensitive to moisture stress as evident from a progressive decrease in germination with an increase in mean germination time at osmotic potential (**Table 2**). Maximum germination in both the seeds was recorded in control with complete inhibition at osmotic potential of $\alpha \leq -0.6$ MPa. At -0.4 MPa, brownish-black seeds took six days to start germination in comparison to cream seeds in which germination started on 3rd day of incubation. Osmotic potential of -0.4 MPa reduced the germination of cream seeds by 57 percent points with 2.2 fold increase in mean germination time than control. Whereas brownish-black seeds recorded 62 percent point reduction in germination with a 3 fold increase in mean germination time as compared to control. Moisture stress exhibited a more pronounced effect on brownish-black seeds than cream seeds. The osmotic potential required for 50% inhibition of the maximum germination of cream and brownish-black seeds was -0.37 and -0.32 MPa, respectively (**Figure 2a and b**). The cream and brownish-black seedlings grown at osmotic potential -0.4 MPa recorded 79.2 and 88.2% reduction in seedling vigour index I as compared to their respective controls (**Figure 5c**).

Bargali and Bargali (2016) reported that population of *M. denticulata* from the Himalayan region of India recorded a decrease in germination from 51 to 10% as the water stress level increased from 0 to -1.0 MPa with complete inhibition at -1.5 and - 2.0 MPa. In contrast to this, germination of our population was completely inhibited even at osmotic potential of -0.6 MPa which indicates that different biotypes of the same weed have differential tolerance to moisture stress. The results of our study suggest that germination of brownish-black seeds would be more adversely affected by moisture stress.

Table 2. Effect of moisture stress on germination of cream and brownish-black seeds of *M. denticulata*

Osmotic potential (MPa)	Germination (%)		Time to start germination(days)		Germination speed		Mean germination time (days)	
	C	B	C	B	C	B	C	B
0 (Control)	95.5	85.5	2.0	3.0	10.9	9.9	3.0	3.1
-0.1	84.4	80.0	2.0	3.0	9.4	8.8	3.3	3.4
-0.2	78.8	73.3	2.0	3.0	8.4	7.8	3.5	3.6
-0.4	38.8	23.3	3.0	6.0	3.6	2.8	6.6	9.3
-0.6	NG	NG	NG	NG	NG	NG	NG	NG
-0.8	NG	NG	NG	NG	NG	NG	NG	NG
-1.0	NG	NG	NG	NG	NG	NG	NG	NG
LSD (p=0.05)	5.98	6.38	1.4×10^{-5}	1.4×10^{-5}	0.09	0.17	0.14	0.09

C-Seeds with cream seed coat; B-Seeds with brownish-black seed coat; LSD-Least significant difference; NG-No germination



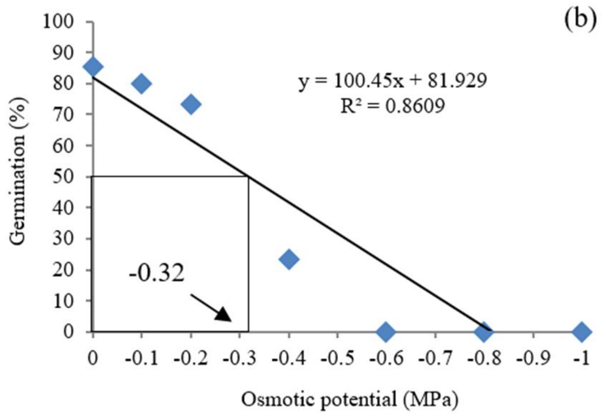


Figure 2. Osmotic potential required for 50% inhibition of maximum germination in (a) seeds with cream seed coat and (b) seeds with brownish-black seed coat. Osmotic potential required for 50% inhibition of germination is shown by an arrow

Tolerance of cream seeds to moisture stress is consistent with their dormancy characteristics whereas brownish-black seeds are more sensitive to moisture stress. The differential response to tolerate moisture stress conditions of heteromorphic seeds might be a survival mechanism in *M. denticulata* under adverse environmental conditions.

Effect of salinity

Salinity stress caused a significant decrease in germination and speed of germination with concomitant increase in mean germination time in both cream and brownish-black seeds (**Table 3**). Increasing NaCl concentrations above 50 mM was more detrimental to germination of brownish-black seeds than cream seeds. At 150 mM NaCl concentration, germination of cream seeds was decreased by 73.3% with a 2.8 fold increase in mean germination time than control. Whereas brownish-black seeds recorded 78.9% reduction in germination with a 2.8 fold increase in mean germination time as compared to control. The NaCl concentration required for 50% inhibition of maximum germination for cream and brownish-black seeds was 100 and 70.6 mM NaCl, respectively (**Figure 3a and b**). Cream seeds were fairly tolerant to salinity stress as some seeds (10%) were able to germinate up to NaCl concentration of 200 mM in contrast to brownish-black seeds whose germination was completely inhibited at 200 mM NaCl. Seedling vigour index of cream and brownish-black seedlings was reduced by 91.1 and 95.6% at NaCl concentration of 150 mM (**Figure 5d**).

Salinity is an important abiotic factor affecting seed germination. It reduces both germination rates as well as root growth of seedlings. The ability to withstand saline conditions may vary from species to species. Guan *et al.* (2009) reported that germination of *M. ruthenica* was maximum in control (100%) with >80% germination at NaCl concentration of 50 and 100 mM. However, salinity stress of 200 mM declined the germination to 55%. In contrast to this, in the present study germination of both cream and brownish-black seeds of *M. denticulata* was <50% at 100 mM with complete inhibition at NaCl concentration of 250 and 200 mM, respectively. These results clearly show that seeds of *M. denticulata* irrespective of their seed coat colour are more sensitive to salinity stress as compared to *M. ruthenica*. Yao *et al.* (2010) exposed heteromorphic seeds (brown and black) of *C. album* to salinity and found that brown seeds were non-dormant and more

Table 3. Effect of sodium chloride (NaCl) on germination of cream and brownish-black seeds of *M. denticulata*

NaCl (mM)	Germination (%)		Time to start germination (days)		Germination speed/index		Mean germination time (days)	
	C	B	C	B	C	B	C	B
0 (Control)	95.5	85.5	2.0	2.0	10.7	9.8	3.1	3.2
25	80.0	73.3	2.0	2.0	9.8	8.6	3.5	3.7
50	72.2	62.2	3.0	3.0	8.6	7.3	3.9	4.3
75	63.3	46.6	3.0	4.0	7.2	6.2	5.2	4.9
100	46.6	20.0	4.0	4.0	3.8	4.4	7.6	6.5
150	22.2	6.6	5.0	5.0	2.3	3.4	9.1	9.1
200	10.0	NG	6.0	NG	0.3	NG	10.1	NG
250	NG	NG	NG	NG	NG	NG	NG	NG
LSD (p=0.05)	5.61	5.92	1.3×10^{-5}	1.3×10^{-5}	0.16	0.10	0.19	0.15

C-Seeds with cream seed coat; B-Seeds with brownish-black seed coat; LSD-Least significant difference; NG-No germination

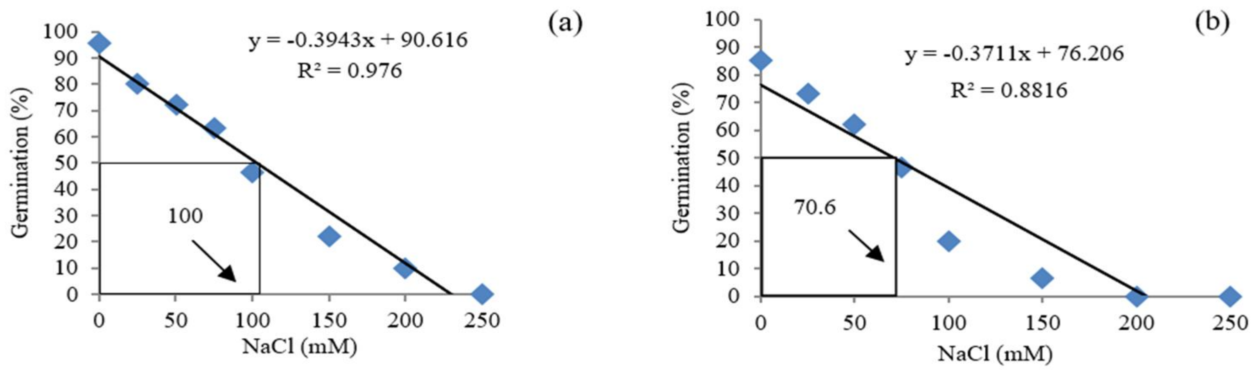


Figure 3. NaCl concentration required for 50% inhibition of maximum germination in (a) seeds with cream seed coat and (b) seeds with brownish-black seed coat of *M. denticulata*. Sodium chloride concentration required for 50% inhibition of germination is shown by an arrow

tolerant of salinity as compared to black seeds which were salt sensitive and a large proportion of seeds was dormant. In contrast to this, the results of our study demonstrate that non-dormant brownish-black seeds were more sensitive to salinity as compared to cream seeds which were dormant and showed increased tolerance against salinity.

Effect of pH

The highest germination of both cream and brownish-black seeds was recorded in control having pH 6.6 and lowest at pH 3 (**Table 4**). However, both seeds were able to germinate under both acidic and alkaline pH with >40% germination in pH range 3-10 which implies that germination of both seeds is not likely to be limited by soil pH. However, at pH 3, germination of brownish-black seeds was reduced to 46.6% as compared to cream seeds with more than 50% germination. This indicates that cream seeds are more tolerant to acidic pH. The maximum time to start germination in both cream and brownish-black seeds was recorded at pH 3 and 4 indicating that acidic conditions delayed the onset of germination. At pH 3, the germination index of both cream and brownish-black seeds was minimum and the mean germination time was longest. The highest values of seedling vigour index I for both cream and brownish-black seeds were recorded in control and lowest at pH 3 **(Figure 5e)**.

Bullitta *et al.* (1994) reported that favorable growth of *M. polymorpha* is usually restricted to soils with a pH of 4.7-8. Graziano *et al.* (2010) reported that *M. polymorpha* is well adapted to alkaline soils. However, it has also been shown to grow on

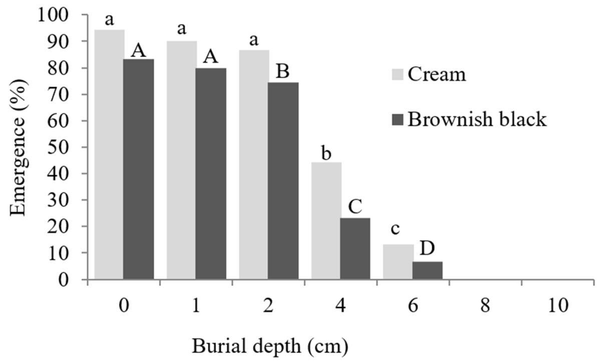


Figure 4. Effect of burial depth on emergence of cream and brownish-black seeds of *M. denticulata*. Columns with same lower case (cream) and upper case letters (brownish-black) do not differ significantly at 5% level of significance

Table 4. Effect of pH on germination of cream and brownish-black seeds of *M. denticulata*

pH	Germination (%)		Time to start germination (days)		Germination speed		Mean germination Time (days)	
	C	B	C	B	C	B	C	B
Control (6.6)	92.2	83.3	2.0	2.0	10.8	9.8	3.0	3.3
3	55.5	46.6	4.0	4.0	6.0	5.2	6.1	7.2
4	60.0	53.3	4.0	4.0	6.4	5.9	5.7	6.2
5	63.3	58.8	2.0	2.0	8.5	8.9	3.7	4.7
6	83.3	76.6	2.0	2.0	10.2	9.3	3.3	3.5
7	86.6	78.8	2.0	2.0	10.5	9.5	3.1	3.4
8	80.0	74.4	2.0	2.0	10.4	9.3	3.5	3.5
9	80.0	72.2	2.0	2.0	10.3	9.1	3.4	3.6
10	76.6	68.8	2.0	2.0	9.8	8.4	3.6	4.1
LSD (p=0.05)	4.66	4.54	$1.3{\times}10^{-5}$	$1.3{\times}10^{-5}$	0.18	0.24	0.26	0.25

C-Seeds with cream seed coat; B-Seeds with brownish-black seed coat; LSD-Least significant difference moderately acidic soils due to its tolerance to acidic conditions. In the present study also, germination of both cream and brownish-black seeds occurred in acidic pH range of 3-5. The pH of agricultural lands in Punjab varies from 7 to 8. In this pH range, *M. denticulata* possessed 80-86% germination, indicating that pH is not likely to be a limiting factor for the germination of this weed.

Effect of burial depth

The maximum emergence of both cream and brownish-black seeds was recorded from surface placed seeds (**Figure 4**). There was a progressive decline in emergence of both seeds with an increase in burial depth from 1-4 cm. At 4 cm depth, the emergence of cream and brownish-black seeds was reduced by 52 and 60 percent points as compared to surface placed seeds. The emergence of cream and brownish-black seeds was 13.3 and 6.6%, respectively at burial depth of 6 cm. No emergence was observed from seeds placed at a depth of 8 cm or deeper. The requirement of light and limited availability of storage reserves are major constraints for the reduced emergence of weeds from deeper soil layers (Bullied *et al.* 2012).

Results of our study indicate that germination of both cream and brownish-black seeds of *M. denticulata* was independent of light enabling the weed to emerge from soil depths of 6 cm owing to the bigger seed size (1000 seed weight of cream and brownish-black seeds was 3.56 and 3.65 g respectively). However, small-seeded species like *Poa annua* (1000 seed weight = 0.3 g) and *R. dentatus* (1000 seed weight = 2.33 g) may not have enough energy reserves to support their emergence from deeper soil depths which could be responsible for their emergence only from shallow soil depths (less than 3 cm).

Conclusion

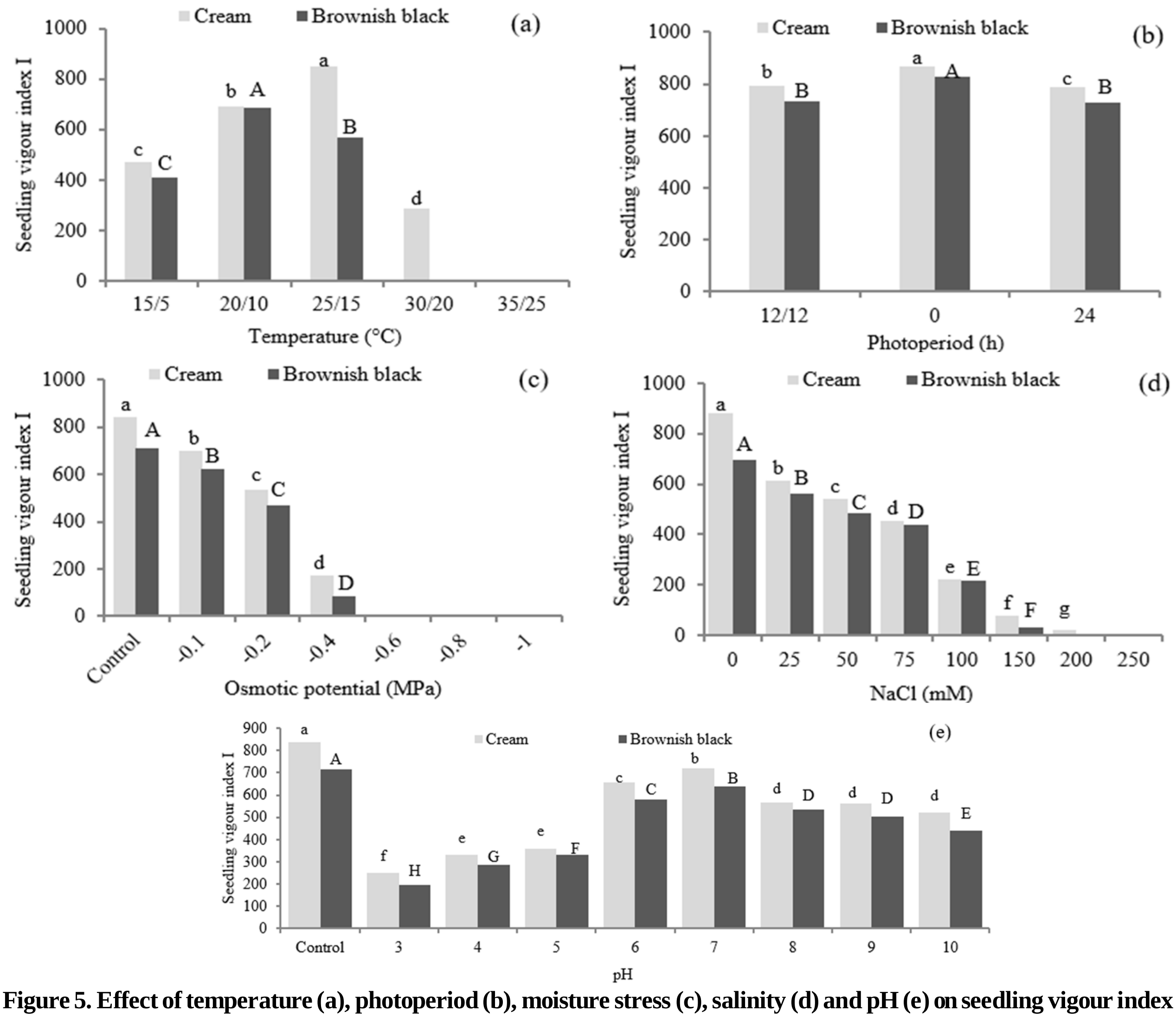


Figure 5. Effect of temperature (a), photoperiod (b), moisture stress (c), salinity (d) and pH (e) on seedling vigour index I of *M. denticulata*. Columns with same lower case (cream) and upper-case letters (brownish-black) do not differ significantly at 5% level of significance

Cream seeds of *M. denticulata* can germinate in a broad range of temperatures whereas germination of brownish-black seeds can occur only in a narrow temperature range. At 30/20°C, germination of brownish-black seeds is completely inhibited and cream seeds exhibited 54.4% germination. Germination of cream as well as brownish-black seeds was independent of light which indicates that both seeds might have an equal chance of germination when present on the soil surface or buried in the soil profile. However, seeds buried to 6 cm or greater depths are most likely to get their food reserves exhausted before the emergence of seedlings. The study also indicates that cream seeds of *M. denticulata* possessed a greater ability to tolerate salinity and moisture stress as compared to brownish-black seeds. Seed germination over a broad pH range (3-10) indicates that pH is not a limiting factor for germination of cream and brownish-black seeds.

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