Variation in the Breeding System of Prunella vulgaris L.

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Variation in the Breeding System of Prunella vulgaris L.

Abstract

*Prunella vulgaris* (Lamiaceae), commonly known as selfheal, is a perennial herb with a long history of use in traditional medicine. Recent studies have found that *P. vulgaris* possesses anti-inflammatory, antiviral, and antibacterial properties, and it is likely that this will lead to increased commercial demand for this species. To date, research publications on *P. vulgaris* cultivation and genetics are scarce. Using accessions originally collected from different geographical regions, we investigated the breeding system of this species by observing variation in floral morphology, time of pollen release, and selfed-seed set in bagged flowers and isolated plants. Two types of floral morphology, one with exerted styles, extending past open corollas when viewed from above, and the other with shorter, inserted styles, were found among 30 accessions. Two accessions originally collected from Asia uniformly displayed exerted styles, and 27 accessions had inserted styles. One accession from Oregon displayed variation in this trait among individual plants. Microscopic observation of seven accessions, including ones with both exerted and inserted styles, revealed that they all release pollen to some degree before the flowers open. Using bagged flowers, we found that selfed-seed set varied widely among eight accessions, ranging from 6% to 94%. However, bagging may underestimate seed set for some accessions. The two accessions with the lowest rates when using bagged flowers increased in seed set by 350% and 158%, respectively, when we evaluated single, unbagged plants in isolation cages. The accession with 6% selfed-seed set when bagged also had exerted styles. These findings suggest that mating systems in *P. vulgaris* may be in the process of evolutionary change and that understanding breeding-system variation should be useful in developing efficient seed-regeneration protocols and breeding and selection strategies for this species.

Keywords
floral morphology, in-bud pollen release, selfed-seed set, bud autogamy

Disciplines
Agricultural Science | Agriculture | Agronomy and Crop Sciences | Horticulture | Plant Breeding and Genetics | Plant Sciences

Comments
This article is from *HortScience* 46, no. 5 (May 2011): 688–692.

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Variation in the Breeding System of Prunella vulgaris L.

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Additional index words. floral morphology, in-bud pollen release, selfed-seed set, bud autogamy

Abstract. Prunella vulgaris (Lamiaceae), commonly known as selfheal, is a perennial herb with a long history of use in traditional medicine. Recent studies have found that P. vulgaris possesses anti-inflammatory, antiviral, and antibacterial properties, and it is likely that this will lead to increased commercial demand for this species. To date, research publications on P. vulgaris cultivation and genetics are scarce. Using accessions originally collected from different geographical regions, we investigated the breeding system of this species by observing variation in floral morphology, time of pollen release, and selfed-seed set in bagged flowers and isolated plants. Two types of floral morphology, one with exerted styles, extending past open corollas when viewed from above, and the other with shorter, inserted styles, were found among 30 accessions. Two accessions originally collected from Asia uniformly displayed exerted styles, and 27 accessions had inserted styles. One accession from Oregon displayed variation in this trait among individual plants. Microscopic observation of seven accessions, including ones with both exerted and inserted styles, revealed that they all release pollen to some degree before the flowers open. Using bagged flowers, we found that selfed-seed set varied widely among eight accessions, ranging from 6% to 94%. However, bagging may underestimate seed set for some accessions. The two accessions with the lowest rates when using bagged flowers increased in seed set by 350% and 158%, respectively, when we evaluated single, unbagged plants in isolation cages. The accession with 6% selfed-seed set when bagged also had exerted styles. These findings suggest that mating systems in P. vulgaris may be in the process of evolutionary change and that understanding breeding-system variation should be useful in developing efficient seed-regeneration protocols and breeding and selection strategies for this species.

Breeding systems play crucial roles in the evolutionary dynamics of plant species (Charlesworth, 2006), are critical in the development of effective and efficient genetic improvement strategies (Charlesworth, 2006; Poehlman, 1987), and should not be overlooked in _ex situ_ germplasm conservation (Sackville Hamilton et al., 2003). Variation in plant sexual breeding systems ranges from cleistogamy and in-bud pollination, mechanisms to ensure self-pollination, to mechanisms that foster cross-pollination such as dioecy and self-incompatibility. However, many plant species vary between these two extremes in their reproductive biology (Goodwillie et al., 2005).

_Prunella vulgaris_ L. (Lamiaceae), commonly known as selfheal, is a low-growing perennial herb. Its dried inflorescences have a long history of use in traditional Chinese and European medicine (Mattioli, 1586; Pinkas et al., 1994) as a remedy for sore throat, fever, and wounds. Recent studies have found that this species has potential to become an economically important medicinal herb through its anti-inflammatory, antiviral, and antibacterial activities (Brindley et al., 2009; Chiu et al., 2004; Kageyama et al., 2000; Psotová et al., 2003; Zhang et al., 2007). With the emergence of such information, it is likely that this will lead to increased commercial demand for this species. Several investigations of seed germination in _P. vulgaris_, its typical method of horticultural propagation, have recently been published in response to new interest in its cultivation (Guo et al., 2009a, 2009b, 2009c; Zhang et al., 2008, 2009). Therefore, studies to optimize commercial production and initiate genetic improvement are likely to follow.

_Prunella vulgaris_ is native to a large part of the Northern Hemisphere. Pioneering ecological research documented significant variation in plant-growth patterns and flowering phenology (Bocher, 1949; Nelson, 1967), typically considered to be the result of plant adaptation to particular habitats or climatic conditions. Recent research has demonstrated that there is significant variation in antiviral properties among different _P. vulgaris_ accesses cultivated under a common set of field conditions (Brindley et al., 2009). Eleven such accessions were also shown to differ more than 10-fold in their concentration of the bioactive compound, rosmarinic acid (Berhow et al., personal communication) in dried, aboveground parts collected at peak flowering. These results strongly suggest that there is substantial genetic variation among populations of this species, at least for phytochemical properties. This variation is likely to confer differences in pharmacological efficacy.

To date, research publications on _Prunella_ cultivation and genetics are scarce. Other than the Chinese germination studies cited previously, most recent research on _P. vulgaris_ has focused on analyses of its chemical constituents and medicinal properties (e.g., Brindley et al., 2009; Han et al., 2009; Psotová et al., 2003; Zhang et al., 2007). This species has also been used as a model plant for ecological and evolutionary research through detailed studies of traits such as seed size and degree of clonal propagation and geographic analyses of hybridity (Fritsche and Kaltz, 2000; MacK and Lepš, 2003; Schmid, 1985; Winn, 1985, 1987; Winn and Cross, 1993). Although it has been stated that _P. vulgaris_ is outcrossing, but self-compatible, and the presence of “cleistogamy” in smaller-flowered plants has been noted (Nelson, 1964, 1967), we are aware of no in-depth investigations on this subject.

_Prunella vulgaris_ was recently incorporated into the medicinal plant germplasm collection conserved by the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS, Ames, IA) and from 2007 to 2011 has been one of three medicinal-plant genera being studied in Iowa by the Center for Research on Botanical Dietary Supplements (2010). For the purpose of establishing effective and efficient seed regeneration methods and to guide its future crop improvement for producers and end-users, we conducted a series of observations and experiments designed to elucidate its breeding system initiated when we observed interesting variation in patterns of floral morphology and senescence.

Materials and Methods

_Prunella vulgaris_ plants used in this investigation were grown from seeds of accessions originally collected from different geographical regions (Table 1), except for Ames 29994 and 29995, which were received as plants from Quarryhill Botanical Garden, Glen Ellen, CA, where they had been grown isolated from each other because the original collections were made in China and Japan in 1990 and 1989, respectively. Additional information about the origins of these accessions is available online.
through the Germplasm Resources Information Network (GRIN) database at <http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?29826>. The seed lots we used had been regenerated in screened field cages at the NCRPIS with honeybees supplied in the cages to promote cross-pollination (Widrlechner et al., 1997) and were all one generation removed from the original collections. For germination, seeds were placed on water-saturated blotter paper in clear, plastic germination boxes held in a germinator, depending on the accession and temperature conditions. All digital photographs were taken between 1000 and 1100 h. Our interest in investigating the possibility of in-bud pollen release began when we observed that anthers and pollen from newly opened flowers of plants of an accession collected in the Republic of Georgia (Ames 29157) seemed atypically old as judged by color and microscopic examination (data not shown), and the flowers wilted less than 8 h after opening. These observations led us to suspect that pollen might be released before the flowers open. To evaluate this characteristic, inflorescences with well-developed but unopened flowers from plants of seven accessions representing a broad range in geographic origin and floral morphology (marked by asterisks in Table 1) were used in this investigation. Inflorescences from two or three plants of each accession were bagged with waxed paper bags (Lawson SB217; Lawson Bag Company, Northfield, IL) to exclude honeybees or other insects. Plants of seven of these accessions were grown in screened cages with introduced pollinators (following Widrlechner et al., 1997) in the field at the NCRPIS. Inflorescences of the eighth accession (Ames 29995) were bagged on plants grown in containers in the greenhouse. We bagged inflorescences that had no open flowers but did have flowers that we expected to open in the next 1 to 2 d. After placing an inflorescence into the bag, the open end of the bag was stapled shut around the stem to secure it in place. Ten d after bagging the inflorescences, the bags were checked every 2 to 3 d to document floral and fruit development by removing and replacing the bags quickly. After the last flower senesced, the top end of the pollination bag was removed. The resulting infructescences were harvested when the sepals had consistently turned a brownish yellow color. Seeds were fully ripe at this stage. Seed counts were made for each flower, which normally produce a maximum of four seeds (nutlets). For

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Origin</th>
<th>Exserted style</th>
<th>Inserted style</th>
<th>No. of flowers bagged and (unbagged)†</th>
<th>No. of seeds set bagged and (unbagged)†</th>
<th>Proportion of maximum seed set (%)‡</th>
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<tbody>
<tr>
<td>Ames 27748</td>
<td>Iowa</td>
<td>✔️</td>
<td></td>
<td></td>
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<tr>
<td>Ames 28312</td>
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<td></td>
<td></td>
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<td></td>
</tr>
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<td>✔️</td>
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<td></td>
<td>332</td>
<td>1117</td>
<td>84</td>
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<tr>
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<td></td>
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<td></td>
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<tr>
<td>Ames 28959</td>
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<td>✔️</td>
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<td></td>
<td>599</td>
<td>1067</td>
<td>45</td>
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<tr>
<td>Ames 29048</td>
<td>Oregon</td>
<td>✔️</td>
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<td></td>
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</tr>
<tr>
<td>Ames 29155</td>
<td>Rep. Georgia</td>
<td>✔️</td>
<td></td>
<td>212</td>
<td>260</td>
<td>31</td>
</tr>
<tr>
<td>Ames 29156</td>
<td>Rep. Georgia</td>
<td>✔️</td>
<td></td>
<td>238 (108)</td>
<td>814 (360)</td>
<td>86 (83)</td>
</tr>
<tr>
<td>Ames 29157</td>
<td>Rep. Georgia</td>
<td>✔️</td>
<td></td>
<td>415 (473)</td>
<td>1370 (1892)</td>
<td>83 (82)</td>
</tr>
<tr>
<td>Ames 29158</td>
<td>Rep. Georgia</td>
<td>✔️</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ames 29159</td>
<td>Rep. Georgia</td>
<td>✔️</td>
<td></td>
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</tr>
<tr>
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<td></td>
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<tr>
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</tr>
<tr>
<td>Ames 29162</td>
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<tr>
<td>Ames 29232</td>
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<td>✔️</td>
<td></td>
<td>446</td>
<td>554</td>
<td>31</td>
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<tr>
<td>Ames 29662</td>
<td>Missouri</td>
<td>✔️</td>
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<tr>
<td>Ames 29663</td>
<td>Illinois</td>
<td>✔️</td>
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<tr>
<td>Ames 29664</td>
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<td>✔️</td>
<td></td>
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</tr>
<tr>
<td>Ames 29994</td>
<td>Sichuan, China</td>
<td>✔️</td>
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<tr>
<td>Ames 29995</td>
<td>Japan</td>
<td>✔️</td>
<td></td>
<td>198</td>
<td>49</td>
<td>6</td>
</tr>
<tr>
<td>PI 656838</td>
<td>Iowa</td>
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<tr>
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<td>✔️</td>
<td></td>
<td>230</td>
<td>865</td>
<td>94</td>
</tr>
<tr>
<td>PI 656841</td>
<td>Iowa</td>
<td>✔️</td>
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</tr>
<tr>
<td>PI 656842</td>
<td>Missouri</td>
<td>✔️</td>
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</tr>
</tbody>
</table>

†Seed-set when plant populations were grown in isolation cages where cross-pollination was promoted by supplying honeybees.
‡Maximum seed-set for a single flower is four for Prunella vulgaris.
§Accession evaluated for in-bud pollen release.
two accessions from the Republic of Georgia, Ames 29156 and 29157, we also harvested mature, unbagged infructescences that had been visited by honeybees and assessed their seed production to serve as a control treatment.

**Selfed-seed set evaluation by unbagged flowers in isolation cages.** Because levels of seed set may be influenced by bagging the flowers when compared with flowers that are displayed under natural self-pollination conditions (Kearns and Inouye, 1993), in 2010, we grew single plants in isolation cages to evaluate selfed-seed production without bagging. The two accessions with the lowest levels of selfed-seed set under bagged conditions (Ames 29995 and Ames 29049) were selected for this experiment. Twenty plants (10 from each accession) were grown in separate cages along with flowering plants from families other than Lamiaceae with nucleus hives of honeybees supplied to facilitate pollen movement. Seeds of five randomly chosen infructescences from each singly caged plant were harvested at seed maturity. For comparable controls, seeds of five randomly chosen infructescences from each of 10 cross-pollinated plants of the two accessions grown together in single regeneration cages were also harvested at seed maturity.

**Results**

**Floral morphology and in-bud pollen release.** We observed striking variation in floral morphology when viewing open flowers from above. Plants of the two accessions originally collected from Japan and China uniformly displayed exerted styles that can be noticed immediately after flowers open and that extended ≥ 2 mm beyond the upper portion of the corolla (Fig. 1, top row) when observed 6 h to 1 d later. Twenty-seven other accessions uniformly presented flowers with styles well covered by the corollas and could not be seen overhead (Fig. 1, bottom row). Within-accession variation in this floral trait was found among plants of one accession, Ames 29049 from Oregon. Among the six plants observed, we found one with flowers displaying exerted styles (Fig. 2).

Our observations with a dissecting microscope found that in-bud pollen release was occurring in all seven accessions evaluated (Fig. 1C). A flower of *P. vulgaris* contains four dimorphic stamens, two short and two long. Each anther has two pollen sacs, connected at the base, which dehisce from the upper side releasing abundant, white pollen grains. For some accessions with inserted styles, for example Ames 29157, pollen grains deposited on the stigmas were clearly observed when the unopened flower buds were dissected (Fig. 3), whereas in other cases, we only found the pollen grains adhering to the surface of the anthers or to the inside of the corolla tube.

**Selfed-seed set by bagged flowers.** Seed set from bagged infructescences was found to vary widely among the eight accessions we evaluated (Table 1). High rates of selfed-seed sets for two accessions from the Republic of Georgia resembled seed-set percentages observed in unbagged, control infructescences that had been visited by honeybees in the isolation cages (Table 1). The accession with the lowest selfed-seed set was Ames 29995 from Japan, which produced only 6% seed set from bagged flowers and consistently displayed exerted styles. Notably, bagged flowers of the single plant of Ames 29049 that we observed with exerted styles also did not produce seeds.

**Seed set comparison of bagged flowers, unbagged singly caged plants, and cross-pollinated plants.** Selfed-seed set with unbagged flowers was higher than with bagged flowers but lower than that observed from cross-pollinated flowers for both accessions (Table 2). Compared with bagged flowers, unbagged flowers produced 3.5× more selfed seed in Ames 29995 and greater than 1.5× more in Ames 29049. As expected, cross-pollinated flowers produced the most seed set for both accessions.

**Discussion**

Nelson (1964) reported cleistogamy in small-flowered plants of *P. vulgaris* such as those of subsp. *vulgaris* in populations found in California. In our investigations, we found that of these 30 accessions, none has flowers that remain closed all the time, even those from the Republic of Georgia, which produced the smallest flowers. Therefore, complete cleistogamy, in the classic sense, was not detected in these accessions. It is possible that Nelson used this term to describe the phenomenon of in-bud fertilization, which has also been described as “bud-autogamy” (Noormets and Olson, 2006) or “preanthesis cleistogamy,” in which “self-pollination occurs in the bud, followed by anthesis and opportunities for outcrossing” (Culley, 2007).

Other than Nelson’s (1964) note on cleistogamy in *P. vulgaris*, pollen release before the flowers open has not been reported previously in this species. Because this occurred in all seven accessions evaluated, it is very likely in-bud pollen release is widespread in various populations of this species. However, in-bud pollen release did not result in effective selfed-seed set in some accessions (Table 1),

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**Fig. 2. Within-population variation of floral morphology of Prunella vulgaris Ames 29049, with exerted styles (A) and inserted styles (B).**

**Fig. 3. Pollen deposition on stigmas before flowers open in Prunella vulgaris Ames 29157 with arrow indicating pollen deposition on the stigma before the flower opened and inset showing bud stage.**
and for accession Ames 29995 (with only 6% and 23% selfed-seed set for bagged and unbagged flowers, respectively), high levels of self-incompatibility or a similar phenomenon may be operating, as recently observed in another genus in the Lamiaceae, Syncolos-temon (Ford and Johnson, 2008). Additional research is needed to determine the conditions that are required to proceed from in-bud pollen release to stigmatic deposition and, ultimately, to effective self-fertilization.

In Vaccinium myrtilloides Michx., Noorients and Olson (2006) reported that 18% of the flower buds were pollinated before full bloom. It is not clear what percentage of the flowers have been self-pollinated in P. vulgaris before they open. The high rate of self-seed production of accessions that originated from the Republic of Georgia and from Iowa indicates that these accessions are highly self-compatible. Because our evaluation was conducted under the condition of no competition with pollen from different genotypes of the same species because of bagging or isolation-cage conditions, it would be interesting to use genetic markers to determine the proportion of selfing under the conditions of bee-mediated pollination among mixed populations in isolation cages (Widrlechner et al., 1997) for these highly self-compatible accessions. Should these accessions still display very high proportions of self-pollination, this information would be very useful in developing efficient seed-regeneration protocols and breeding and selection strategies for these accessions.

The higher levels of selfed-seed set for unbagger flowers than for bagged flowers for two accessions with low seed set from bagged flowers (Table 2) were expected. Three factors could play contributing roles (Kearns and Inouye, 1993). Environmental conditions for pollination inside the pollination bags could be less favorable as a result of increased temperature and/or humidity or the calm conditions inside the bags could restrict pollen movement within and among flowers and therefore reduce the chances for pollen deposition on the stigmas. These factors could also interact. We suspect that lack of pollen movement is more likely to play an important role, because the unbagger flowers were exposed to large numbers of honeybees, which would promote pollen transfer, but the relative contributions of these factors remain to be quantified.

However, it should also be pointed out that the negative influence of bagging on seed set may be accession-dependent. The two accessions from Republic of Georgia (Ames 29156 and Ames 29157) had similarly high rates of seed set from the bagged flowers when compared with in-cage, cross-pollinated flowers (Table 1). The two Iowa accessions, Ames 29335 and PI 656840, produced 84% and 94%, respectively, selfed seed from bagged flowers. This suggests that bagging likely has a minimal impact on seed production, at least for these accessions. All these accessions have the pollen sacs of the two longer anthers and the stigma presented at a similar level. The bagging effect on lowering selfed-seed set in Ames 29995 could be caused by its exerted styles. Because this may affect pollen deposition to the stigma within a flower or among flowers under calm conditions within the bags.

The relatively low levels of seed set (63%) for cross-pollinated plants of Ames 29995 may also be the result of self-incompatibility. This accession was received from the donor in the form of 47 plants, many of which might have been clones resulting from layering or division during the years that it was being maintained in a public garden or closely related seedlings resulting from consanguineous mating.

Variation in selfed-seed set and the occurrence of exerted styles among these accessions suggests that the mating system in P. vulgaris may be geographically structured or in the process of evolutionary change. If one takes the geographic origins of these accessions into consideration, a potential difference may exist between the high and low self-seed-set accessions. The accessions with lower selfed-seed set (less than 50%) were all originally from the Pacific Rim, and the other accession with exerted styles was from China, whereas high selfed-seed-set accessions (greater than 80%) were from the Republic of Georgia or from Iowa. This geographic pattern may be analogous to the wild tomato relative, Solanum pimpinellifolium L., in which centrally located populations exhibit floral traits that promote outcrossing, but peripheral populations are highly autogamous (Rick et al., 1977, 1978; Zuriaga et al., 2009), which may be related to effective range expansion and colonization. If that is the case, this would suggest that P. vulgaris originated in eastern Asia and then expanded its range across the northern hemisphere. The evaluation of more Prunella accessions from a wider geographic range will be required to determine if such a relationship is operating or if our observation is just an artifact of small sample size.

Our investigations reveal that the breeding system in P. vulgaris is more complex than previously thought. The results presented herein can help direct more in-depth studies of its breeding system and suggest strategies for effective genetic conservation and crop improvement. For example, differences in breeding systems among populations of S. pimpinellifolium were also observed in genebank accessions. Based on those observations, Widrlechner (1987) discussed the practical implications of using different seed-regeneration methods for its conservation. For Prunella accessions that effectively produce seeds through in-bud pollination, but not after flowers open, protected seed regeneration in cages as currently practiced at the NCRPIS would not be needed. However, other accessions such as those presenting exerted styles (e.g., Ames 29995) may require insect vectors for good seed production. From a crop-improvement perspective, the development of pure lines may be quite straightforward in accessions that exhibit a high degree of in-bud autogamy, whereas those that do not may present much higher levels of within-accession diversity and be useful for the creation of F1 hybrids. Using observations of pollen-tube growth to evaluate possible mechanism(s) of self-incompatibility and artificial pollination to document outcrossing differences among accessions would produce more in-depth knowledge on the breeding system of P. vulgaris and also be useful for genetic improvement efforts.

**Table 2. Seed set comparison of bagged flowers, unbaggered, singly-caged plants and cross-pollinated plants for two Prunella vulgaris accessions.**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Bagged</th>
<th>Unbaggered</th>
<th>Cross-pollinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ames 29049</td>
<td>31(±23)</td>
<td>49(±5)</td>
<td>88(±2)</td>
</tr>
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<td>Ames 29156</td>
<td>24(±4)</td>
<td>23(±5)</td>
<td>63(±7)</td>
</tr>
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</table>

**Literature Cited**


