Variation in the Breeding System of *Prunella vulgaris* L.

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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 eco-

logical 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* accessions 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; Macek 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

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Table 1. Flower morphology and seed set of different *Prunella vulgaris* accessions.

Genotype	Origin	Flower morphology		No. of flowers bagged and (unbagged) ^z	No. of seeds set bagged and (unbagged) ^z	Proportion of maximum seed set (%) ^y
		Exerted style	Inserted style			
Ames 27748	Iowa		✓			
Ames 28312	Iowa		✓			
Ames 28314	Iowa		✓			
Ames 28355	Iowa		✓			
Ames 28356	Iowa		✓			
Ames 28358	Iowa		✓	332	1117	84
Ames 28359	Iowa		✓			
Ames 28435	Iowa		✓			
Ames 28959	Iowa		✓			
Ames 29047 ^x	Oregon		✓	599	1067	45
Ames 29048	Oregon		✓			
Ames 29049 ^x	Oregon	✓	✓	212	260	31
Ames 29155	Rep. Georgia		✓			
Ames 29156	Rep. Georgia		✓	238 (108)	814 (360)	86 (83)
Ames 29157 ^x	Rep. Georgia		✓	415 (473)	1370 (1892)	83 (82)
Ames 29158	Rep. Georgia		✓			
Ames 29159	Rep. Georgia		✓			
Ames 29160	Rep. Georgia		✓			
Ames 29161	Rep. Georgia		✓			
Ames 29162	Rep. Georgia		✓			
Ames 29232 ^x	Oregon		✓	446	554	31
Ames 29662	Missouri		✓			
Ames 29663	Illinois		✓			
Ames 29664	Illinois		✓			
Ames 29994 ^x	Sichuan, China	✓				
Ames 29995 ^x	Japan	✓		198	49	6
PI 656839 ^x	Iowa		✓			
PI 656840	Iowa		✓	230	865	94
PI 656841	Iowa		✓			
PI 656842	Missouri		✓			

^zSeed-set when plant populations were grown in isolation cages where cross-pollination was promoted by supplying honeybees.^yMaximum seed-set for a single flower is four for *Prunella vulgaris*.^xAccession evaluated for in-bud pollen release.

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 (Widrechner 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 germination chamber at 25/30 °C (16 h/8 h) under continuous fluorescent light. Germination usually took place in 5 to 14 d. Seedlings were transplanted into flats and grown in the NCRPIS greenhouse before field planting.

Observations of floral morphology and in-bud pollen release. Floral morphology was evaluated through visual inspection, and flowers were digitally photographed for a permanent record. Observations focused on finding any obvious differences in the arrangement of reproductive parts that might be related to the mating system. All 30 accessions (Table 1) were examined during the flowering period. Twenty-five or more plants were examined for each accession except for accession Ames 29049, which had only six plants as a result of damage during the preceding winter. Flowers of *P. vulgaris* first open in the morning from 0500 HR to 1000 HR and typically remain open for 8 to 36 h or longer, depending on the accession and temperature

conditions. All digital photographs were taken between 1000 and 1100 HR.

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 collected in the field between 0730 and 0830 HR on 17 July 2009. Inflorescences from each accession were placed into covered plastic cups containing a thin layer of water to prevent wilting. These inflorescences were brought to the laboratory, and pollen release was examined with a dissecting microscope (Nikon SMZ1500; Nikon USA, Melville, NY) equipped with a digital camera (Nikon DS-5M) linked to a computer and a display monitor. Unopened flower buds were removed from the inflorescence and placed on the microscope stage. The upper portion of the corolla was carefully removed with a pair of tweezers and a dissecting needle to expose the anthers and the

style without disturbing their positions. Digital photomicrographs were then taken and stored.

Selfed-seed set evaluation by bagging flowers. In 2009, plants from eight accessions (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 Widrechner 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

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 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/inner 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

Fig. 1. Flower types and in-bud pollen release of *Prunella vulgaris*. Top row: Ames 29995 [(A) flowers with exerted styles, (B) stage for observation of in-bud pollen release, and (C) in-bud pollen release]. Bottom row: PI 656839 [(A) flowers with inserted styles, (B) stage for observation of in-bud pollen release, and (C) in-bud pollen release].

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 $\times$ more selfed seed in Ames 29995 and greater than 1.5 $\times$ 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).

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.

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),

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

Accession	Seed set ($\pm$ SD) (%)		
	Bagged	Unbagged	Cross-pollinated
Ames 29049	31($\pm$ 23)	49($\pm$ 5)	88($\pm$ 2)
Ames 29995	6($\pm$ 4)	23($\pm$ 5)	63($\pm$ 7)

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, *Syncolostemon* (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., Noormets 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 selfed-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 (Widrechner 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 unbagged 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 unbagged 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 29358 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 selfed-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, Widrechner (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 F_1 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.

Literature Cited

- Bocher, T. 1949. Racial divergences in *Prunella vulgaris* in relation to habitat and climate. *New Phytol.* 48:285–314.
- Brindley, M.A., M.P. Widrechner, J.A. McCoy, P. Murphy, C. Hauck, L. Rizshsky, B. Nikolau, and W. Maury. 2009. Inhibition of lentivirus replication by aqueous extracts of *Prunella vulgaris*. *Virology* 6:8. 8 Jan. 2010. <<http://www.virologyj.com/content/6/1/8>>.
- Center for Research on Botanical Dietary Supplements. 2010. Research. Iowa State University, Ames, IA. 22 Feb. 2010. <<http://www.nwrc.iastate.edu/botanical/research/>>.
- Charlesworth, D. 2006. Evolution of plant breeding systems. *Curr. Biol.* 16:R726–R735.
- Chiu, L.C., W. Zhu, and V.E. Ooi. 2004. A polysaccharide fraction from medicinal herb *Prunella vulgaris* downregulates the expression of herpes simplex virus antigen in Vero cells. *J. Ethnopharmacol.* 93:63–68.
- Culley, T. 2007. The cleistogamous breeding system: A review of its frequency, evolution, and ecology in angiosperms. *Bot. Rev.* 73:1–30.
- Ford, C.M. and D. Johnson. 2008. Floral traits, pollinators and breeding systems in *Syncolostemon* (Lamiaceae). *Plant Syst. Evol.* 275:257–264.
- Fritsche, F. and O. Kaltz. 2000. Is the *Prunella* (Lamiaceae) hybrid zone structured by an environmental gradient? Evidence from a reciprocal transplant experiment. *Amer. J. Bot.* 87: 995–1003.
- Goodwillie, C., S. Kalisz, and C.G. Eckert. 2005. The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. *Annu. Rev. Ecol. Syst.* 36:47–79.
- Guo, Q., L. Liu, R. Zhao, and Y. Kong. 2009a. Studies on germination characteristics of *Prunella vulgaris* seeds. *China J. Chinese Materia Medica* 31:1045–1047.
- Guo, Q., X. Zhang, X. Shen, and Y. Chen. 2009b. Effects of seed priming on drought tolerance in *Prunella vulgaris*. *China J. Chinese Materia Medica* 34:1195–1198.
- Guo, Q., X. Zhang, Y. Wang, L. Liu, and Y. Chen. 2009c. Study on seed quality test and quality standard of *Prunella vulgaris*. *China J. Chinese Materia Medica* 34:812–816.
- Han, E.H., J.H. Choi, Y.P. Hwang, H.J. Park, C.Y. Choi, Y.C. Chung, J.K. Seo, and H.G. Jeong. 2009. Immunostimulatory activity of aqueous extract isolated from *Prunella vulgaris*. *Food Chem. Toxicol.* 47:62–69.

- Kageyama, S., M. Kurokawa, and K. Shiraki. 2000. Extract of *Prunella vulgaris* spikes inhibits HIV replication at reverse transcription in vitro and can be absorbed from intestine in vivo. *Antivir. Chem. Chemother.* 11:157–164.
- Kearns, C.A. and D.W. Inouye. 1993. Techniques for pollination biologists. Univ. Press of Colorado, Niwot, CO.
- Macek, P. and J. Lepš. 2003. The effect of environmental heterogeneity on clonal behaviour of *Prunella vulgaris* L. *Plant Ecol.* 168:31–43.
- Mattioli, P.A. 1586. Kreuterbuch. Verlegung Sigmund Feyerabends, Peter Fischers, und Heinrich Dacken, Frankfurt am Main.
- Nelson, A. 1964. Relationships between two subspecies in a population of *Prunella vulgaris* L. *Evolution* 18:43–51.
- Nelson, A. 1967. Racial diversity in Californian *Prunella vulgaris*. *New Phytol.* 66:707–746.
- Noormets, M. and A.R. Olson. 2006. Bud-autogamy in the velvet-leaf blueberry, *Vaccinium myrtilloides* Michx. *Can. J. Plant Sci.* 86:245–250.
- Pinkas, M., F. Trotin, M. Feng, and M. Torck. 1994. Use, chemistry and pharmacology of the Chinese medicinal plants. *Fitotherapia* 55:343–353.
- Poehlman, J.M. 1987. Breeding field crops. 3rd Ed. AVI Publishing, Westport, CT.
- Psotová, J., M. Kolár, J. Soušek, Z. Švagera, J. Vičar, and J. Ulrichová. 2003. Biological activities of *Prunella vulgaris* extract. *Phytother. Res.* 17:1082–1087.
- Rick, C.M., J.F. Fobes, and M. Holle. 1977. Genetic variation in *Lycopersicon pimpinellifolium*. Evidence of evolutionary change in mating systems. *Plant Syst. Evol.* 127:139–170.
- Rick, C.M., M. Holle, and R.W. Thorp. 1978. Rates of cross-pollination in *Lycopersicon pimpinellifolium*: Impact of genetic variation in floral characters. *Plant Syst. Evol.* 129:31–44.
- Sackville Hamilton, R., J. Engels, and T. van Hintum. 2003. Considerations for improved conservation and utilization concepts and strategies, p. 43–59. In: Engels, J.M.M. and L. Visser (eds.). A guide to effective management of germplasm collections. IPGRI Handbook for Genebanks #6. IPGRI, Rome, Italy.
- Schmid, B. 1985. Clonal growth in grassland perennials III. Genetic variation and plasticity between and within populations of *Bellis perennis* and *Prunella vulgaris*. *J. Ecol.* 73:819–830.
- Widrechner, M.P. 1987. Variation in the breeding system of *Lycopersicon pimpinellifolium*: Implication for germplasm maintenance. *FAO/IBPGR Plant Genetic Resources Nws.* 70:38–43.
- Widrechner, M.P., C.A. Abel, and R.L. Wilson. 1997. Ornamental seed production in field cages with insect pollination. *Combined Proc. of the IPPS* 46:512–516.
- Winn, A.A. 1985. The effects of seed size and microsite on seedling emergence of *Prunella vulgaris* in four habitats. *J. Ecol.* 73:831–840.
- Winn, A.A. 1987. Regulation of seed yield within and among populations of *Prunella vulgaris*. *Ecology* 68:1224–1233.
- Winn, A.A. and K.L. Cross. 1993. Latitudinal variation in seed weight and flower number in *Prunella vulgaris*. *Oecologia* 93:55–62.
- Zhang, X., Q. Guo, and X. Shen. 2009. Effects of seed priming on salt tolerance in *Prunella vulgaris* seed germination under saline conditions. *China J. Chinese Materia Medica* 34: 944–947.
- Zhang, X., Q. Guo, and Y. Wang. 2008. Effects of seed priming on vigor of *Prunella vulgaris* seeds. *China J. Chinese Materia Medica* 33:493–495.
- Zhang, Y., P.P.H. But, V.E.C. Ooi, H.X. Xu, G.D. Delaney, S.H.S. Lee, and S.F. Lee. 2007. Chemical properties, mode of action, and in vivo anti-herpes activities of a lignin-carbohydrate complex from *Prunella vulgaris*. *Antiviral Res.* 75:242–249.
- Zuriaga, E., J.M. Lanca, L. Cordero, A. Sifres, W.G. Blas-Cerdán, R. Morales, and F. Nuez. 2009. Genetic and bioclimatic variation in *Solanum pimpinellifolium*. *Genet. Resources Crop Evol.* 56:39–51.