

Research paper

Use of bridge species and rescue pollination for introducing the wild diploid 1EBN species germplasm to cultivated potatoes

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Abstract

The commercial potato is an autotetraploid with a narrow genetic base, while wild diploid potato species are sources of genetic diversity and genes for resistance to most of the biotic and abiotic stresses along with quality traits. However, the utilization of wild species in potato breeding is restricted by pre- and post-zygotic crossing barriers resulting from different ploidy levels, endosperm balance number, or genomic structure. Diploid 1EBN species, due to such barriers, are considered the tertiary germplasm for potato breeding. Here we used *Solanum verrucosum*, a rare self-compatible wild diploid potato, as a bridge species along with rescue pollination and indolebutyric acid (IBA) application for germplasm transferring from four 1EBN species including *S. commersonii*, *S. bulbocastanum*, *S. cardiophyllum*, and *S. pinnatisectum*. Results showed that rescue pollination for fruit retention seems slightly better than the IBA application. The same results were also obtained for the seed set. IBA mainly led to fully parthenocarpic fruits. Seeds obtained from these crosses had gibberellic acid resistance dormancy and low germination capacity. As it was expected, the crossability of *S. commersonii* with *S. verrucosum* was better than the other three species. Interspecific *S. verrucosum* × *S. commersonii* hybrids showed both male and female fertility. However, they were crossable with the cultivated diploid potato species *S. phureja* just as the female parent.

Keywords: auxin; crossing barrier; endosperm balance number; potato; wild diploid potato species

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Introduction

The commercial potato is an autotetraploid species. Despite having many wild relatives, commercial potato cultivars have a narrow genetic base. So, potato breeders are interested to use wild relatives to widen its genetic base by introducing desirable traits from these relatives. Wild potatoes exist in five cytological groups including 2x, 3x, 4x, 5x, and 6x. The majority (73%) of tuber-bearing species of *Solanum* are diploids (Hawkes

1994; Muthoni *et al.* 2012; Hosaka and Sanetomo 2020).

The crossability between potato species is complicated. For instance, some diploid potatoes are easily crossed with the cultivated potatoes and unexpectedly most of the progenies of such crosses are tetraploid instead of triploid due to the 2n gamete function. However, there are some other diploid species that are not cross-compatible not only with the tetraploids but also with the mentioned diploid

species. Based on such results, Johnston *et al.* (1980) proposed the endosperm balance number (EBN) hypothesis for the explanation of the crossing behavior of potato species. Based on this hypothesis, each species has an effective ploidy or EBN that may be different from its chromosomal ploidy, and the cross will be successful if the 2:1 ratio of maternal to paternal EBN exists in the endosperm. In other words, for a cross to be successful, the parental species must have equal EBN. The EBN is assigned to the *Solanum* species based on the crossing behavior of the species in the cross with the standard species (Ortiz and Ehlenfeldt 1992; Jackson and Hanneman 1999; Machida-Hirano 2015). Accordingly, diploid potatoes were classified into two groups of 1 and 2 EBN species. These two groups are reproductively isolated from each other. The 2EBN diploid species include cultivated diploids and many other South American wild diploid species and the 1EBN group includes mainly Mexican and a few South American diploid potatoes (Ortiz and Ehlenfeldt 1992; Jackson and Hanneman 1999). Most of the diploid 2EBN species can be crossed to the cultivated diploid and also the dihaploids of the cultivated tetraploids as well as commercial cultivars via $2n$ gametes but the use of 1EBN species in potato breeding is challenging and needs a tedious ploidy manipulation methods or protoplast fusion techniques.

Solanum verrucosum is a rare $2x$, 2EBN species from Mexico, and unlike most other diploid 2EBN species it is self-compatible and lacks mRNase in its pistil (Ortiz and Mihovilovich 2020). Hanneman (cited by Jansky and Hamernik, 1999) proposed that this species may be a suitable candidate as a bridge species for transferring the 1EBN species germplasm to the cultivated potato.

This study aimed to broaden the genetic base of the potato in Iran, using the genes of wild 1EBN species. Here we report the use of *S. verrucosum* as the bridge parent for introgressing the germplasm of the wild 1EBN species in the 2EBN background, which is easily crossable with the cultivated potato.

Material and Methods

The plant material used in this study was listed in Table 1. The seeds of *verrucosum* and four 1EBN species were obtained from the CGN gene bank of The Netherlands, but the clone from the *phureja* species was developed in Department of Horticultural Science, University of Tabriz, Iran by sib-mating among the progeny of the cross [(GLK 1497 \times CGN 17669) \times IvP 48], a haploid inducer clone with the embryo spot-marker gene. The seeds of these species were planted in the cell trays containing the perlite and cocopeat as the substrate in the polyhouse. When seedlings reached the 4-leaf stage, they were

transplanted to individual pots with the same substrate.

For enhancing the flowering and berry setting, all of the female parents and most of

the male parents were grafted on the tomato rootstock. All of the plants during the crossing period were fertigated by the Hogland nutrient solution.

Table 1. The potato species used in the study, their ploidy level, EBN, and the abbreviated name.

Parent	Species	Ploidy and EBN
Male	<i>Solanum bulbocastanum</i>	2x, 1EBN
	<i>Solanum cardyophyllum</i>	2x, 1EBN
	<i>Solanum commersonii</i>	2x, 1EBN
	<i>Solanum pinnatisectum</i>	2x, 1EBN
Female	<i>Solanum verrucosum</i>	2x, 2EBN
Rescue pollinator	<i>Solanum phureja</i>	2x, 2EBN

Crossing

The flower buds of female parents 1 day before opening were emasculated by removing the petals and anthers carefully and a day after they are pollinated by freshly harvested pollens of male parents. Although we tried to use fresh pollen some pollinations were also made by using stored pollens.

One day after pollination with the pollens of the target species some of the pollinated flowers were pollinated with the pollens of 2x, 2EBN genotypes carrying the embryo spot marker gene as rescue pollination, and the others were treated by the 15 ppm of indolebutyric acid (IBA).

The berry set was recorded in each group. Berries were retained for 4 weeks on the plant and after they were harvested and maintained in the laboratory until softening, and then seeds were extracted.

Extracted seeds after some month's storage were treated with 1000 ppm GA₃ and

then planted between moistened filter paper in a Petri dish and after germination transferred to cell trays.

Ploidy determination of seedlings at first judged by chloroplast counting in guard cells of the leaf lower epidermis but finally approved by chromosome counting. For this root tips from young growing roots were dissected and then treated with 0.1 M 8-hydroxyquinolin for 2 h and fixed in 3:1 (ethanol: acetic acid) after that root tips were washed with distilled water, hydrolyzed in 1N HCl at 60 °C for 10 min and then stained with 1% aceto orcein. Microscopic slides prepared by squash method.

Data analysis

Data for the comparison of rescue pollination and IBA effect on fruit set and seed number were done by chi square test. For comparison of 1EBN species (male parents), data for each species were pooled (rescue pollination and

IBA treatment) and analyzed by the Kruskal-Wallis test and then the means were compared by the Tukey's non-parametric test.

Results and Discussion

Table 2 shows the results from the crossing of *S. verrucosum* as the female with the three Mexican 2x, 1EBN (*S. cardiophyllum*, *S. bulbocastanum*, and *S. pinnatisectum*) and one South American 2x, 1EBN (*S. commersonii*) as the male parents, followed by the rescue pollination or application of the plant growth regulators. The rescue pollination for fruit retention and seed set seems slightly better than the IBA application (Table 2). For the fruit set there was a significant difference between the rescue pollination and IBA application in *S. bulbocastanum* and *S. pinnatisectum* and for the seed set these differences were significant in *S. commersonii*, *S. bulbocastanum*, and *S. cardiophyllum* (Table 2). Among the 1EBN species, *S. commersonii* was better than other species for fruit set, number of seeds per 100 pollinations, and number of seeds per 100 fruits (Table 3). Although all of the male species used in these crosses were 1EBN, geographically, *S. commersonii* is of South American but *S. bulbocastanum*, *S. pinnatisectum*, and *S. cardiophyllum* are of Mexican origin and considered primitive species compared to most of the South American potato species (Hawkes 1991). Therefore, based on the cytogenetical

studies it is expected that the *commersonii* genome is close to *verrucosum* and the cultivated potatoes (Hawkes 1994). Dinu *et al.* (2005) made many crosses of *S. verrucosum* and dihaploids of the cultivated potato with the 1EBN species and its doubled-chromosome counterparts along with applying the plant growth regulators and the embryo rescue. They didn't get any hybrids without using the embryo rescue even when they used the doubled-chromosome forms of the 1EBN species that had the compatible EBN number. Also, in some crosses, the seeds had no germination capacity. Jansky and Hamernik (2009) by making 1389 crosses between *verrucosum* species and four 1EBN species, including *S. cardiophyllum*, *S. chancayense*, *S. commersonii*, and *S. trifidum* obtained 17 interspecific inter-EBN hybrids, which majority of them belonged to *S. verrucosum* × *S. commersonii*. While Yermishin *et al.* (2014) reported exceptional results from the crosses *S. verrucosum* × 1EBN species. They obtained 1000 seeds from approximately 100 crosses by using the rescue pollination; their cross efficiency increased to 3600 seeds from 136 pollinations. Our result was very close to Jansky and Hamernik's (2009) results. However, in both of the aforementioned studies, many diverse accessions of *verrucosum* were used and the *S. verrucosum* parent mainly improved the crossing success. All of the hybrids obtained from the *S.*

verrucosum × 1EBN species in this study were diploid (Figure 1b). This was confirmed at first by the chloroplast counting in the guard cells of the lower epidermis of the leaves (Figure 1a) (Ordonez *et al.* 2017) and finally by the chromosome counting in roots or stolon tip

cells' mitosis (Ordonez *et al.* 2017). In the crosses involving the parents with similar ploidy levels but different EBN, the homoploid hybrids were unusual because EBN acts as a powerful screener for the function of 2n gametes from the low-EBN parent side.

Table 2. Results of interspecific crosses between *Solanum verrucosum* as the female parent and 2x 1EBN species, followed by the rescue pollination or IBA application.

1EBN species	Treatment	Percent of fruit set	Number of seeds per100 fruit
<i>Solanum commersonii</i>	Rescue pollinator	39	31
	IBA ⁺	37	22
		Chi-square= 0.175 P= 0.676	Chi-square= 0.175 P= 0.676
<i>Solanum pinnatisectum</i>	Rescue pollinator	46	13
	IBA	32	11
		Chi-square= 4.92 P= 0.045	Chi-square= 2.3 P= 0.065
<i>Solanum bulbocastanum</i>	Rescue pollinator	50	4
	IBA	33	0
		Chi-square= 3.55 P= 0.041	
<i>Solanum cardyophyllum</i>	Rescue pollinator	38	14
	IBA	37	7
		Chi-square= 5.9 P= 0.082	Chi-square= 3.4 P= 0.035

IBA: Indolebutyric Acid

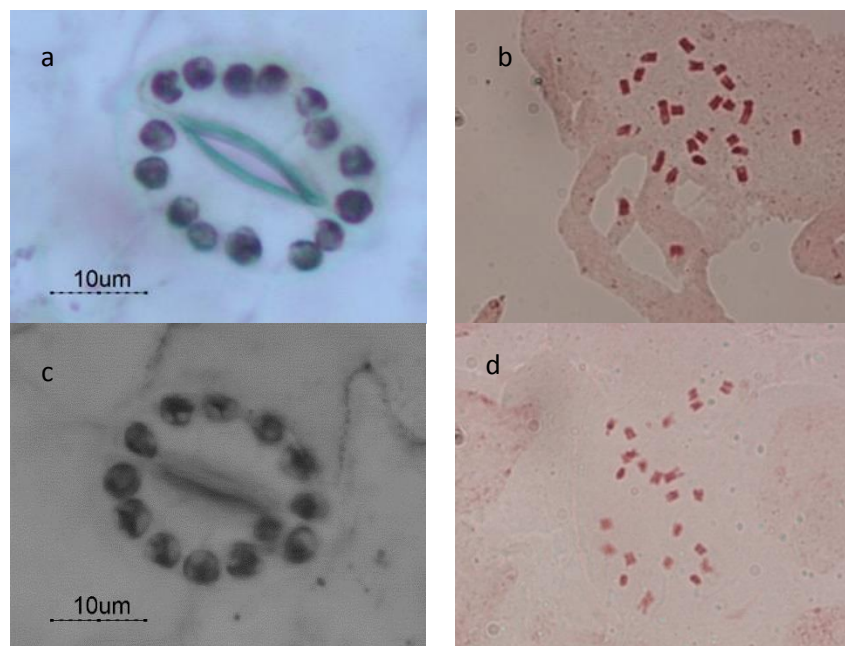


Figure 1. Ploidy determination in the hybrids between *Solanum verrucosum* and 1EBN species. a) Chloroplast number in the stomata guard cells (15), b) Chromosome number ($2n = 2x = 24$), c) Chloroplast number, and d) Chromosome number in *S. phureja* ($2x$) as the control.

However, despite observing some $2n$ pollens in the pollen samples of the 1EBN species (Figure 2), all rare hybrids were diploid. Dinu *et al.* (2005) also couldn't get hybrids from the crosses between the diploid 2EBN species and the doubled-chromosome counterparts of the diploid 1EBN species ($4x$, 2EBN). Novy and Hanneman (1991) selected the $2n$ pollen-producing genotypes of the 1EBN species for crossing with the *S. tuberosum* dihaploids (2EBN) but had very low success. It must be pointed out that the function of $2n$ pollens in the $2x$, $2EBN \times 2x$, and 1EBN will lead to triploid hybrids which are expected to be sterile or with very low fertility. Using them in

repeated backcrossing needs to double their chromosome number, which may limit the recombination rate due to the preferential pairing. However, the diploid hybrids are more efficient in germplasm transferring because it is expected that they will be fertile unlike the triploids, and also provide an opportunity for intergenomic recombination in the F_1 interspecific hybrids.

The $2x$ hybrids obtained from *S. verrucosum* \times *S. commersonii* were relatively vigorous morphologically and partially intermediate between the parental species. All of them were easily crossable as the female with *S. commersonii* with enough seed

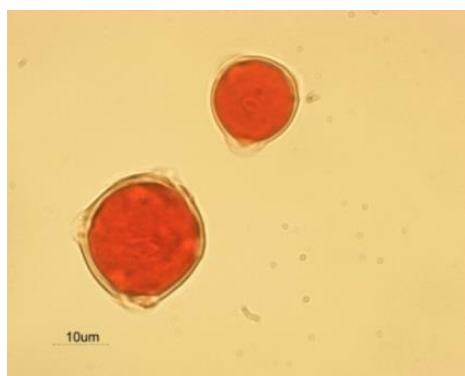


Figure 2. Normal reduced ($1n$, upper) and unreduced ($2n$, lower) pollens in *Solanum cardyophyllum*.

Table 3. The comparison of the 1EBN species in cross with *Solanum verrucosum* based on the fruit set, number of seeds per 100 pollinations and berries.

Species	Percent of fruit set	Number of seeds per 100 pollinations	Number of seeds per 100 berries
<i>Solanum commersonii</i>	71a	16a	23a
<i>Solanum pinnatisectum</i>	41b	5b	12b
<i>Solanum bulbocastanum</i>	44b	2c	3c
<i>Solanum cardyophyllum</i>	19c	6b	11b
(Kruscal - Wallis test)	**	*	**

Means with different letters in each column are significantly different at 5% probability level (The nonparametric Tukey test).

*, **: Significant at 5% and 1% probability levels, respectively.

production because of good germination capacity. But its cross with the diploid-2EBN cultivated species (*S. phureja*) was not easy. However, by doing some efforts, the author obtained some seeds with good germination capacity from all of the crosses of *S. verrucosum* × *S. commersonii* with *S. phureja* (data not shown).

S. commersonii by having some interesting traits such as frost tolerance, resistance to soft rot, and resistance to potato virus X with the best cold acclimatization feature, is one of the important species for potato breeders. Carputo *et al.* (1997, 2000) used chromosome doubling and ploidy manipulation for introducing the soft root resistance and cold hardiness of *S. commersonii* to the cultivated potatoes. They crossed the doubled-chromosome clones of *S. commersonii* (4x, 2EBN) with the cultivated diploid plants (2x, 2EBN) and obtained triploid hybrids. These triploid hybrids by the functioning of the 2n gametes were crossed with the commercial cultivars (4x, 4EBN) and yielded complex pentaploid hybrids, that when backcrossed to the cultivated parents, yielded tetraploid or near tetraploid progenies. However, this method is more time-consuming, and also its success depends on the formation of the 2n gamete. The use of *S. verrucosum* as the bridge species for producing diploid hybrids between *S. verrucosum* × *S.*

commersonii is a shorter and less tedious way than the ploidy manipulation for the germplasm transferring. Based on Hawkes (1994), the genomes of *S. commersonii* and *S. verrucosum* are not much differentiated from each other, so it is expected that in these hybrids the recombination occurred between *S. commersonii* and *S. verrucosum* genomes. Especially, the diploid hybrids will provide more opportunities for inter-specific recombination than the triploid hybrids because in the triploid hybrids, preferential pairing may limit the intergenomic pairing and recombination. Also, 2x progenies are more suitable for use in breeding programs with recently introduced diploid hybrids (Jansky *et al.* 2016; Alpers and Jansky 2019).

Most of Iran's potato production areas are located in the northwest, which most times is prone to frost damage in early spring. Therefore, the *S. verrucosum* × *S. commersonii* hybrids obtained here could be used for introducing the frost tolerance from *S. commersonii* to the commercial cultivars. Especially now the backcross progeny of *S. verrucosum* × *S. commersonii* hybrids with the diploid cultivated species (*S. phureja*) are available.

Conclusion

In this research, we used *S. verrucosum* as a bridge species and the rescue pollination

technique for introducing the germplasm of wild 1EBN diploid species to the diploid cultivated 2EBN background. Interspecific *S. verrucosum* × *S. commersonii* hybrids as female parents produced a few seeds after pollinating with the pollens of *S. phurja*, a cultivated 2x, 2EBN species. The results indicated that the use of *S. verrucosum*, as the bridge species, and rescue pollination is the most effective way for using the wild diploid 1EBN species germplasm in potato breeding, especially for the development of diploid hybrid cultivars.

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Conflict of Interest

The authors declare that they have no conflict of interest with any people or organization concerning the subject of this manuscript.

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انتقال ژرم پلاسما گونه‌های دیپلوئید IEBN به سیب زمینی زراعی با استفاده از گونه پل و گرده افشانی نجات دهنده

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چکیده

سیب زمینی تجاری گونه اتوتتراپلوئید با پایه ژنتیکی محدود می‌باشد در حالی که گونه‌های دیپلوئید وحشی منبع تنوع ژنتیکی و واجد ژن‌های مقاومت به اغلب تنش‌های زیستی و غیرزیستی همراه با ژن‌های مربوط به خصوصیات کیفی هستند. اما کاربرد گونه‌های وحشی در اصلاح سیب زمینی به دلیل موانع دورگ‌گیری پیش و پس از تشکیل سلول تخم به علت تفاوت در سطح پلوئیدی، عدد توازنی آندوسپرم (EBN) و یا ساختار ژنومی محدود شده است. گونه‌های دیپلوئید IEBN به دلیل چنین موانعی به عنوان خزانه ژنی ثالثیه سیب زمینی در نظر گرفته می‌شوند. در این مطالعه از گونه *Solanum verrucosum*، یک گونه دیپلوئید وحشی خودبارور نادر، به عنوان گونه پل و نیز بکارگیری گرده افشانی نجات دهنده و اکسین (IBA) برای انتقال ژرم پلاسما چهار گونه دیپلوئید وحشی سیب زمینی IEBN (*S. pinnatisectum*، *S. commersonii*، *S. bulbocastanum* و *S. cardiophyllum*) به سیب زمینی زراعی استفاده شد. نتایج نشان داد که گرده افشانی نجات دهنده برای حفظ میوه اندکی بهتر از بکارگیری IBA می‌باشد. همان نتایج برای تشکیل بذر نیز دیده شد و IBA عمدتاً منجر به میوه‌های کاملاً پارتنوکارپ شد. بذرها به دست آمده از تلاقی‌ها دارای خواب مقاوم به ژیرلین بودند و قدرت جوانه زنی چندانی نداشتند. همان طور که انتظار می‌رفت *S. commersonii* در مقایسه با سه گونه دیگر از قدرت تلاقی پذیری بیشتری با *S. verrucosum* برخوردار بود. هیبریدهای بین گونه‌ای *S. verrucosum* × *S. commersonii* از نر باروری و ماده باروری برخوردار بودند. در عین حال تلاقی آن‌ها با سیب زمینی زراعی دیپلوئید (*S. phureja*) تنها به صورت والد ماده امکان پذیر بود.

واژه‌های کلیدی: اکسین؛ سیب زمینی؛ عدد توازنی آندوسپرم؛ گونه‌های دیپلوئید وحشی سیب زمینی؛ موانع دورگ‌گیری