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Assessment of the Possibility of Direct Crossing Between Cultivated Potato and Two Wild Allotetraploid Relatives

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Abstract: The cultivated potato *Solanum tuberosum* L. is an autotetraploid with Endosperm Balance Number (EBN) of 4. There are some allotetraploid 2EBN wild species with desirable traits for potato improvement. However, it is very difficult to cross between these two groups due to differences in EBN. Eleven genotypes of autotetraploid cultivated potato including the six cultivars of subsp. Tuberosum (*tbr*) and five clones of subsp. Andigena (*adg*) were crossed with two wild allotetraploid species: *S. acaule* (*acl*) and *S. stoloniferum* (*sto*). For assess the pollen-pistil incompatibility, some of pollinated flowers were fixed in Carnoy's solution and stained with aniline blue. Evaluation with fluorescent microscope revealed that pre-zygotic pollen-pistil incompatibility in these crosses is frequent. The pistils of both subsp. of cultivated potato were incompatible with pollens of *acl* but when *sto* used as staminate parent it was compatible with five cultivars of subsp. Tuberosum, but incompatible with most of subsp. Andigena (4 clones). From 739, 4x, 4EBN×4x, 2EBN crosses we couldn't get any viable seed. In reciprocal crosses, allotetraploid species were pollinated with a mix of pollens from Tuberosum or Andigena clones. The crosses of *sto*×*adg*, *acl*×*adg* and *acl*×*tbr* were compatible but in the case of *sto*×*tbr* the situation was not clear. From these crosses 157 fruits were obtained but most of them contained only shrink seed and we find just one hybrid from the *sto*×*tbr* combination. The hybrid was tetraploid (2n = 4x = 48) and vigorous with profuse flowering and good fertility. When this hybrid was used as the pistillate parent it was compatible with both parental species.

Key words: *Solanum*, potato, crossing barrier, EBN, wild allotetraploid

INTRODUCTION

The main cultivated potato *Solanum tuberosum* L. is a tetrasomic tetraploid species with two subspecies, *S. tuberosum* subsp. Andigena (*adg*) and *S. tuberosum* subsp. Tuberosum (*tbr*) (Carputo *et al.*, 2003).

There are more than 200 tuber-bearing species in the genus *Solanum* with many valuable traits for potato improvement. Wild potato species are found in a ploidy series ranging from diploid to hexaploid (Hawkes, 1992, 1994). But many of these species have interspecific crossing barriers. These barriers are classified as pre-zygotic barriers like the inhibition of pollen germination or pollen tube growth (stylar barrier) and post-zygotic, such as ploidy barrier and the dysfunction of the hybrid endosperm (Masuelli and Camadro, 1997; Janssen *et al.*, 1997; Jackson and Hanneman, 1999; Chen *et al.*, 2004; Yermishin *et al.*, 2006).

Fifteen percent of the potato species including the cultivated potato are tetraploid but most of them have

disomic inheritance and are called disomic tetraploids (allotetraploid). The most important allotetraploid species for the potato breeding are *S. acaule* (*acl*) and *S. stoloniferum* (*sto*) that belong to series Acaulia and Longipedicellata, respectively. These species can be crossed with most of diploid potatoes without critical problem, but they cannot be crossed directly with tetrasomic tetraploid potatoes which mainly attributed to endosperm imbalance and is controlled by a genetic system called Endosperm Balance Number (EBN). Under the EBN system normal development of the hybrid endosperm requires a 2:1 maternal to paternal EBN ratio in this tissue. So, crosses will be compatible at the endosperm level between species with same EBN, but not between species with different EBN (Masuelli and Camadro, 1997) The EBN was determined for most of *Solanum* species by crossing each with species of known EBN (Hanneman, 1994). Based on the results of such crosses the EBN values of 4 and 2 were assigned to cultivated tetraploid and wild allotetraploid potatoes,

respectively (Hanneman, 1994; Carpato *et al.*, 2003). Therefore, although cultivated potato and its wild allotetraploid relatives have the same ploidy level, direct crosses between them are difficult to achieve due to difference in EBN. In order to circumvent the interspecific incompatibilities due to different EBN values, breeders have used several strategies such as using bridge species along with ploidy manipulation, somatic chromosome doubling of lower EBN parent, selecting the genotypes of the lower EBN parent that produce functional unreduced gametes (2n) and somatic hybridization (Camadro and Espinillo, 1990; Adiwilaga and Brown, 1991; Watanabe *et al.*, 1992; Janssen *et al.*, 1997; Ortiz, 2001; Iovene *et al.*, 2002; Carpato and Barone, 2005; Yermishin *et al.*, 2006). However, these alternative methods can have certain disadvantages in the process of introgression (Watanabe *et al.*, 1992; Janssen *et al.*, 1997; Yermishin *et al.*, 2006).

True tetraploid hybrids from sexual crosses in addition to short time should enhance homoeologous pairing and recombination and therefore facilitate the germplasm introgression from allotetraploid species to cultivated potato, but these interspecific hybrids have been difficult to obtain due to the EBN difference between wild allotetraploid and cultivated tetraploid potato (Watanabe *et al.*, 1992; Janssen *et al.*, 1997). Still several successful inter-EBN hybridizations have been reported (Hermesen, 1994).

Iwanaga *et al.* (1991) and Singsit and Hanneman (1991) reported successful hybridization between cultivated and wild allotetraploids by the use of rescue pollination followed by embryo rescue. Furthermore Janssen *et al.* (1997) claimed that by doing more crosses the production of 4x hybrids between cultivated potato and wild allotetraploid relatives are possible.

Considering the evidence that in inter-EBN crosses *adg* probably have more possibility of producing hybrid progeny than *tbr* (Hanneman and Rhude, 1978), the aim of this study was to attempt to direct crossing the wild allotetraploid with cultivated potato and comparing the two subspecies of *S. tuberosum* through assessing the feasibility of producing hybrid progeny.

MATERIALS AND METHODS

The plant material used in this study was divided in 2 groups.

- The wild allotetraploid (4x, 2EBN) that included 2 species *S. acaule* and *S. stoloniferum*. The *S. acaule* comprised two accessions (CPC 1167 and CGN 17845) and *S. stoloniferum* was a hybrid of CPC 9 × Nijmegen 80470206.

- The cultivated autotetraploid (4x, 4EBN) included 5 clones of *S. tuberosum* subsp. *Andigena* namely *adg*₁, *adg*₂, *adg*₃, *adg*₄, *adg*₅. These clones were selected from a population breeding program in Department of Horticultural Sciences, University of Tabriz (Massiha *et al.*, unpublished data) and were under the three cycle of selection for tuberization under long day condition. The original population was derived from some accessions of GLK and CGN potato gene banks. From *S. tuberosum* subsp. *Tuberosum*, 6 cultivars namely *Agria*, *Aula*, *Diamond*, *Kennbec*, *Satina*, and *Shepody* were used in crosses.

The true seeds of wild species were sown in Petri dishes and seedlings were transplanted into pots. After enough growth scion were taken and grafted on tomato rootstock.

Seed tubers of both subspecies of cultivated potato were planted into pots, after emergence scion were taken from them and grafted on tomato rootstock also.

The grafted plants were then planted in a screen house in Agricultural Research Station of University of Tabriz in 2005. Controlled crosses were performed one day before anthesis, on buds previously emasculated. Pollen for pollination collected as bulk from each species and subspecies. The number of pollinations, fruits and seeds were recorded. The fruits with more than 5 seeds were removed with the assumption that they were originated from unwanted self fertilization. The seeds were treated with 1500 ppm gibberellic acid (GA₃) for 24 h before planting.

Fluorescent microscopic observation of pollen tubes in the styles was done by fixing the pollinated stigma and styles in Carnoy's solution (3 ethanol : 1 glacial acetic acid) 48 h after pollination. Following fixation they were rinsed with distilled water, treated with 5 N sodium hydroxide for approximately 24 h, rinsed with distilled water, and stained with 0.1% aniline blue dye in 0.1N K₃PO₄ with adjusted pH on 9. (Novy and Hanneman, 1991). Styles were then mounted in a drop of dye solution, covered, squashed gently and observed at 100x magnifications with a Nikon fluorescent microscope.

Wild species evaluated for 2n pollen. This was done by staining the pollen with a mixture of 1:1, 1% acetocarmin W/V in 45% V/V acetic acid : glycerol for at least 3 h, pollen grain were observed and counted with 200x magnifications. This method also was used for estimating the male fertility of the hybrid plant. In addition, the pollen samples of the hybrid plant were cultured in media containing 10% sucrose, 1% Agar and 0.01% boric acid.

RESULTS

Forty seven plants (17 from *sto*, 15 *acl* CPC 1167 and 15 of *acl* CGN 17845) were screened for pollen of big size (putative 2n pollen) but 2 n pollen producing plants were not observed. In some plants, there were big pollens in a very low percentage (less than 0.5%), but they were not of practical value.

A total of 739 pollination were made between the *S. tuberosum* as pistillate parent and wild allotetraploid as staminate parent (4x, 4EBN×4x, 2EBN), the result of which are summarized in Table 1. It is obvious that the results of these crossing were not encouraging.

As is indicated in Table 1, the pre-zygotic barriers in the crosses of *tuberosum*×allotetraploid species are frequent. All of the six cultivars of subsp. *tuberosum* involved in this study as pistillate parent in crosses with *acl* were show to be incompatible (Fig. 1a and b). But except the cv. Kennebec other *tbr* genotypes had not pre-zygotic incompatibility with *sto* (Fig. 1c). The occurrence of pre-zygotic incompatibility in the crosses of subsp. *Andigena*×allotetraploid species were more than that's of *tbr* and all of five examined clones had pre-zygotic incompatibility with *acl* and all of five clones except one were incompatible with *sto*. By the way we get just seven fruits which five of them were parthenocarpic and only two

Table 1: Results of 4x, 4EBN × 4x, 2EBN crossing and pollen tube growth in stigma/style

Female	Male	Pollination	Pollen tube growth	Fruits	Seed
<i>S. tbr</i> ssp. <i>Tbr</i>					
cv. Aula	<i>Acl</i>	17	1/3	-	-
	<i>sto</i>	22	Full	-	-
Agria	<i>acl</i>	14	1/3	-	-
	<i>sto</i>	16	Full	-	-
Diamond	<i>acl</i>	21	2/3	-	-
	<i>sto</i>	27	Full	2	0
Kennebec	<i>acl</i>	11	1/3	-	-
	<i>sto</i>	15	1/3	-	-
Satina	<i>acl</i>	51	1/3	-	-
	<i>sto</i>	64	Full	3	0
Shepody	<i>acl</i>	10	1/3	-	-
	<i>sto</i>	32	Full	-	-
<i>S. tbr</i> ssp. <i>Adg</i>					
clone <i>adg</i> ₁	<i>Acl</i>	10	1/3	-	-
	<i>sto</i>	79	Full	2	2
<i>adg</i> ₂	<i>acl</i>	17	1/3	-	-
	<i>sto</i>	19	2/3	-	-
<i>adg</i> ₃	<i>acl</i>	26	1/2	-	-
	<i>sto</i>	118	1/3	-	-
<i>adg</i> ₄	<i>acl</i>	18	1/2	-	-
	<i>sto</i>	44	1/2	-	-
<i>adg</i> ₅	<i>acl</i>	56	1/3	-	-
	<i>sto</i>	52	1/3	-	-
	total	739		7	2

Table 2: Results of 4x, 2EBN×4x, 4EBN crossing and pollen tube growth in stigma/style

Female	Male	Pollination	Pollen tube growth	Fruits	Seeds
<i>S. acl</i> CGN					
17845 Clone 1	<i>adg</i>	14	Full	3	0
	<i>tbr</i>	12	Full	2	0
Clone 2	<i>adg</i>	21	Full	5	2
	<i>tbr</i>	25	Full	2	1
Clone 3	<i>adg</i>	15	Full	1	1
	<i>tbr</i>	15	Full	0	-
<i>S. acl</i> CPC					
1167 Clone 1	<i>adg</i>	34	Full	12	5
	<i>tbr</i>	31	Full	4	2
Clone 2	<i>adg</i>	38	Full	9	11
	<i>tbr</i>	47	Full	5	8
Clone 3	<i>adg</i>	25	Full	9	4
	<i>tbr</i>	30	Full	4	5
total		307		56	39
<i>S. sto</i> Clone1					
	<i>adg</i>	51	Full	39	6
	<i>tbr</i>	34	1/3-full	4	1
Clone 2	<i>adg</i>	23	Full	19	3
	<i>tbr</i>	30	1/3-full	3	4
Clone 3	<i>adg</i>	22	Full	17	3
	<i>tbr</i>	27	Full	1	0
Clone 4	<i>adg</i>	36	Full	12	7
	<i>tbr</i>	31	Full	6	3
Total		254		101	27

seeds were obtained from the cross of *adg*₁×*sto*. Although these seeds were plump and had normal appearance, after treatment with GA₃ they failed to germinate.

Interestingly reciprocal crosses (4x, 2EBN×4x, 4EBN) from the point of fruit and seed set was better than the 4x, 4EBN × 4x, 2EBN crosses and from 561 crosses, 157 fruits were obtained however, most of fruits were parthenocarpic or with shrunken seeds. Planting the limited normal seeds that were obtained from these crosses resulted to just one hybrid from the cross of *sto*×*tbr* and others were originated from unwanted self fertilization (Table 2).

When the wild species used as pistillate parent, the pollen pistil incompatibility were fewer. The pistil of both wild species was compatible with pollens of *adg* as well as *acl*×*tbr* but in the case of *sto*×*tbr* the situation was not clear, and in *sto* clones of 1 and 2 in some specimen the pollen tubes were arrested in first 1/3 of style (Fig. 1d) but in others pollen tube grew until the base of the style.

Description of hybrid plant: The obtained hybrid plant was vigorous with upright growth habit. Its general appearance was closer to wild parents (*sto*), its leaflets were slightly larger and thicker than its wild parent. The flower shape of hybrid plant approximately was intermediate between parents with more resemblances to cultivated ones. (Fig. 2 a and b).

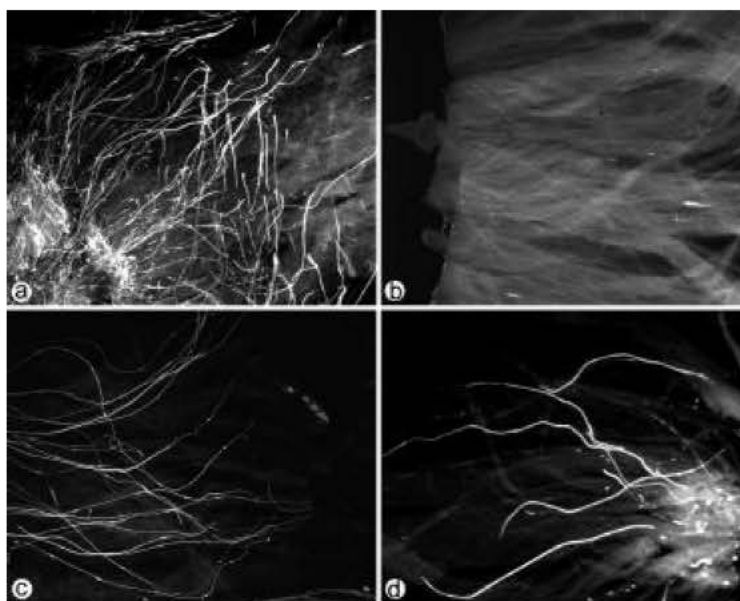


Fig.1: Pollen tube growth and pentrance after 48 h. (a) Most of pollen tube arrested in first 1/3 in cross of Aula × acl. (b) The end of mentioned style in the fig 1. a (c) base of style in cross of Satina × sto with penetrated pollen tube. (d) Pollen tubes arrested in first 1/3 in cross sto × tbr

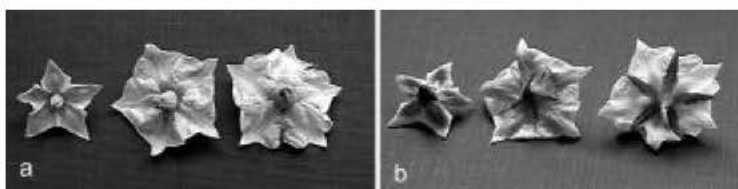


Fig. 2: Flower shape of hybrid (center) and parental species (sto in left, tbr cv. Satina in right). a- Front view of flowers b- Back view of flower note on the calyx shape and lengths in parents and hybrid



Fig. 3: Mitotic metaphase in root tips of hybrid plant indicating $2n = 4x = 48$

Chromosome count was done in the root tips of the stem cutting of the hybrid plant and revealed that it is a tetraploid $2n = 4x = 48$ (Fig. 3)

The male fertility of hybrid as estimated with pollen stainability by acetocarmin jelly was = 35 %, but when assessed by pollen germination on artificial medium it was lower (= 16%). Crosses between the hybrid as male parent with parental species and with diploid 2EBN species was not successful but crosses of the hybrid as pistillate parent was successful and produced viable seeds (data was not shown).

DISCUSSION

Most of the wild potatoes have capability to produce $2n$ pollens; however we could not find any genotype producing $2n$ pollen. This result was predictable since the number of accession examined here was not enough and there is report of this character being rare in allotetraploid species especially in *S. acaule* (Camadro and Espinillo, 1990; Zlesak and Thill, 2002). So we had not possibility to use aforementioned approach of based on selecting plants of wild species that produce $2n$ gamete and

crossing them with cultivated potato to obtain hexaploid progeny with 4EBN which could be used for backcross with cultivated parent. (Camadro and Espinillo, 1990; Ortiz, 2001.).

In plant genera having both self-compatible (SC) and self-incompatible (SI) species the inhibition of SC pollen on SI style commonly occurs; however, in the SI×SI and SC×SC crosses inhibition may also occurs (Hermsen, 1994). The gametophytic self-incompatibility is very frequent in diploid tuber bearing *Solanum*; however, polyploid species such as *S. tuberosum* (both subsp.) as well as wild allotetraploid species are SC due to a phenomenon so called competition interaction (Camadro *et al.*, 2004). Based on Hogenboom (cited by Hermsen, 1994; Masuelli and Camadro, 1997) two concepts describe the genetics of pre-zygotic barrier; 'penetration capacity' of pollen grain includes all genes in the pollen controlling its capacity to overcome barriers to hybridize with alien females and 'barrier capacity' of female parent that includes the genes that control barriers in the pistil against penetration of alien pollen. Our results indicate that *acl* has lower penetration and barrier capacity which is in accordance with results of Masuelli and Camadro (1997) and the subsp. *tbr* in compare with *adg* has lower barriers capacity.

Although the pre-zygotic barrier occurred in most combination but it is obvious that pre-zygotic barrier is not main problem in this kind of crosses. Because there are some crosses without any pre-zygotic barrier such as *Aula*×*sto*, *Agria*×*sto*, *Diamond*×*sto* ... but they give raise only to one hybrid from *sto*×*tbr*. However pollen pistil incompatibility may be a main problem when one how wants to use 2n gamete producing genotypes of 4x, 2EBN in crosses with *tbr* or *adg* especially about *acl* as male parent (Novy and Hanneman, 1991).

Hanneman and Rhude (1978) compared before than EBN theory was offered the crosses of *tbr*×*phureja* (*phu*) with *adg*×*phu* on the frequency of 3x progeny. They found that the frequency of 3x progeny in *adg*×*phu* are more than of that is in *tbr*×*phu* crosses. *phu* is a 2x, 2EBN species, so when it is crossed with 4x, 4EBN in endosperm of 3x embryo the maternal to paternal ratio is 4:1 instead of 2:1 so in contrast to expectation the frequency of 3x progeny are rare. Based on this result it is expected that the subsp. *adg* will be more successful than *tbr* in cross with other 2EBN species such as wild allotetraploids. But our result did not confirm this anticipation. However in our experiment due to the pre-zygotic barrier we could not evaluate the survival of fertilized eggs of *adg* with 1EBN sperm.

Camadro and Masuelli (1995) on the basis of different pairing possibility between the genomes of *acl* proposed a genetic model for EBN. According this model, in *acl* 2.1% of gametes are 2EBN. Under the EBN theory this gametes must be compatible in cross with 4EBN species. Unfortunately *acl* as staminate parent were incompatible in most of crosses, but reciprocal crosses were compatible and 147 crosses with *adg* and 160 with *tbr* as male parent were done but any of these did not result to hybrid offspring, with account of an average 100 eggs in each ovary there are enough opportunity for expressing this 2.1% 2EBN gametes of *acl*. So as it is suggested by Masuelli and Camadro (1997) it seems that EBN is only part of a more complex system of interspecific barriers.

It also appears that obtaining *in vivo* tetraploid hybrid between these two groups of potato (4x, 2EBN and 4x, 4EBN) is chance event and the best method would be the use of rescue pollination followed by embryo rescue as proposed by Iwanaga *et al.* (1991) and Singsit and Hanneman (1991). But with regard to high frequency of pre-zygotic incompatibility it is better at first to asses the pollen pistil compatibility.

Matsubiyashi (cited by Hawkes, 1992) proposed a genome formula of AABB for species in series of Longipedicellata. The genomic formula of *S. tuberosum* is AAAA. Therefore, the obtained hybrid plant must have AAAB genome, in other words it is an autoallotetraploid. There are some reports about the sterility of interspecific hybrids in potato due to the cytoplasmic-genetic interaction and abnormal or even arrested meiosis (Jackson and Hanneman, 1999; Novy and Hanneman, 1991). In spite of its interspecific origin and autoallotetraploidy nature, it had enough male fertility. This may be attributed to its cytoplasm origin (*sto*), because nuclear genes of *sto* like some other wild species interact with cytoplasm of *tbr* resulting to male sterility.

Meiosis of this hybrid is under investigation. It would be informative about the behavior of one set of B genome along with 3A genome.

The hybrid plant must be having of 3EBN (2EBN from *tbr* and one from *sto*). So it must be segregate for EBN and its gametes would be have of EBN value from 1-2, therefore theoretically it could be crossed with both of 2EBN (diploid and allotetraploid) and 4EBN species.

When the hybrid used as staminate parent in cross with its parental species the crosses were not successful presumably due to aneuploidy of male gametes and due to pre-zygotic barriers. But as a female parent it was crossable with both parent and interestingly produced open pollinated fruits. These results confirm its 3EBN nature and segregation for EBN.

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