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Breeding for Nematode and Virus Resistance in Potato via Anther Culture*

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Summary. In *Solanum tuberosum* the production by parthenogenesis of 2x plants with 24 chromosomes, and the regeneration of microspores of such dihaploids to yield monohaploid (1x) plants is reproducibly possible, at least for some specific genotypes. Experiments are described using tissue culture techniques in an applied breeding program with the main aim of increasing the level of resistance to the potato cyst nematode *Globodera pallida* (Stone) and to the potato viruses X, Y and leaf roll. These resistances follow quantitative as well as qualitative modes of inheritance. Using anther culture it is demonstrated that doubled monohaploid clones can be produced which possess the resistance in the homozygous condition. In both ways of inheritance the ratio of resistant clones is rather high. The genotype of the anther donor plant has, however, a strong influence on the total number of androgenetic plants which can be regenerated. Therefore, experiments were initiated with the aim of integrating this capacity for regeneration (tissue culture ability) into valuable genotypes. The results show that the potentiality for regeneration is under genetic control and can be utilized by combination breeding. Its inheritance and physiological basis, as well as the behaviour of complete homozygous clones, is discussed.

Key words: Anther culture – Potato nematodes – Potato viruses – Resistance – Potato breeding

Introduction

The major breeding aim in potato research is to increase the resistance against diseases. Success in this field by using cell and tissue culture should be a convincing example for the potential of the new breeding approach. Of the potato diseases, those caused by viruses, cyst nematodes and *Phytophthora* are the most severe. While Behnke (1979, 1980) reported results on a successful utilization of an in vitro step in increasing the resistance to *Phytophthora infestans* (de By), this paper will concentrate on an examination of the extent with which virus and nematode resistance can be introduced into breeding clones. In a previous paper (Wenzel et al. 1979), we reported on an analytical synthetic breeding scheme, which benefits from the possibility of reducing the tetraploid level of the cultivated potato via the dihaploid level with 2n = 2x = 24 chromosomes to monohaploids (2n = x = 12). Here, experiments were conducted to produce homozygous dihaploid clones possessing resistances, and to broaden the genetic basis for applied in vitro techniques.

In the case of viruses we concentrated on clones carrying the extreme resistance to potato virus X (PVX), which was extracted from *Solanum acaule* (Bitt) and which follows, as a monogenic character, a qualitative mode of inheritance (Ross 1954, 1958), and on field resistance to potato virus Y (PVY) and leaf roll (PLRV), both following a quantitative inheritance. For nematodes, the resistance against the white potato cyst nematode (wPCN), *Globodera pallida*, was investigated. This follows a quantitative mode of inheritance when extracted from *S. vernei* (Bitt. et Wittm.) clones and a more qualitative mode of inheritance when extracted from *S. gourlayi* (Hawkes) (Uhrig and Wenzel 1981). These resistances served in this experimental series as central objectives for incorporation into new and better breeding lines of potato. They also should demonstrate that tissue culture techniques can help to combine valuable characters in crop plants in a more efficient way. The experiments resulted in resistant potato clones which carry the resistance in the homozygous condition. Because of their homozygous nature, the characters will be inherited in a more predictable manner, facilitating a reduction of the population size compared with the classical approach.

* Dedicated to Prof. Dr. Joseph Straub on the occasion of his 70. birthday

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Material and Methods

1 Plant Material

Dihaploid potatoes obtained from tetraploid clones (Baerecke, Frandsen and Ross, unpublished) were utilized as starting material. In order to extend the basis of this material, new dihaploids were also extracted, using the parthenogenetic technique by pollinating with the two *S. phureja* (Juz. et Buk.) clones IvP 35 and IvP 48 (Hermsen and Verdenius 1973). Furthermore, dihaploid hybrids between dihaploid *S. tuberosum* L. and diploid wild species were used. The plants were grown in greenhouses under semicontrolled conditions (20 ± 4°C), where the anther donor plants were routinely grafted on tomato stocks (*Lycopersicum esculentum* (Mill) hybrid 'Supravite') in order to prolong flowering (illumination during the short day season: 10,000 lux, by Osram HQI 1,000 W lamps to give a 16h photoperiod). In addition to greenhouse grown material, the plants were grown in the field at three different locations: 1. Köln-Vogelsang with a high virus pressure because of high aphid populations (up to 1,000 aphids per leaf), 2. Scharnhorst, an aphid poor area, 3. Weidefeld a fairly aphid free area at the shore of the Baltic Sea.

2 Viruses

Experiments were concentrated on resistance to PVX, PVY and PLRV. For all experiments with PVX and PVY, the mechanical inoculation was carried out with mixtures of different virus strains (PVX: X 239, X Jubel; PVY: Y_{vn}, Y_0Y_{1466}). The viruses were maintained in *Nicotiana tabacum* L. ssp. *Xanthi* nc. Crude sap was inoculated using a sponge and carborundum as abrasive. Retesting was performed either on tobacco for PVY or on *Gomphrena globosa* L. for PVX. Tap water was used as dilution agent, if different virus dilutions were applied (Wenzel 1971). In addition to mechanical inoculation the natural virus pressure on the different field locations differentiated the levels of field resistance. PLRV was tested only under this natural infection pressure. The presence of the extreme resistance to PVX was tested by grafting scions of the clones, which should be checked, on PVX infected tomato stocks. After 4 weeks, the scions were retested on *G. globosa*.

3 Nematodes

All experiments were carried out with the white potato cyst nematode (wPCN), *Globodera pallida* (Stone). For artificial infections predominantly the population Chavornay, classified as Pa 3 according to the artificial nomenclature of Kort et al. (1977), was used. Infections and determination of the level of resistance has been described previously (Uhrig and Wenzel 1981). In addition to tests in four chamber bio vessels type K (Behringer 1967) 8cm pots were used in parallel tests. This normally allowed a better growth of the root system and consequently a more correct evaluation. Resistance tests were repeated at least three times. In addition to an identification of resistant and nonresistant clones according to total cyst numbers, P_f/P_i-values smaller than 1.0 for eggs and larvae made it possible to determine the level of resistance to a greater extent.

4 Anther Culture

For all anther culture experiments the method routinely used was as described previously (Sopory et al. 1978; Wenzel et al. 1980). To summarize the most important data: flower buds of 4-6 mm length were harvested and precultured in dry test tubes for two days at 6° C. Then the anthers were dissected and planted on Murashige and Skoog medium (Murashige and Skoog 1962) with a total of 6% sucrose, 0.5% active charcoal and 1 mgl^{-1} 6 BAP (induction medium). The macroscopic structures which develop within 3-4 weeks, were transferred to MS medium supplemented with 10% coconut milk, 0.3 mgl^{-1} zeatin and 3% sucrose (regeneration medium I). As soon as morphogenesis started, the tissue could be transferred to MS medium with 0.5 mgl^{-1} 6 BAP and 3% sucrose (regeneration medium II). When functional shoots were formed these were cut off and transferred as cuttings to the greenhouse.

Ploidy levels were roughly estimated by counting chloroplasts in the stomata or by chromosome counting of very young leaves.

Results

In a previous publication we have reported on the production of 2,000 potato clones with 24 chromosomes regenerated from microspores of dihaploid *S. tuberosum* plants (Wenzel et al. 1979). During the following two years another 4,000 different clones were regenerated, yielding now a total of 6,000 androgenetic (A) clones. About 90% of these, doubled up spontaneously and gave fertile clones with 24 chromosomes directly; only 10% remained monohaploid; a few became tetraploid. The broad phenotypic segregation was a strong indication that the majority of them had passed through meiosis and then had doubled spontaneously to result in completely homozygous clones. For further clarification of this point the nodal band marker was incorporated to enable a judgement for meiotic offspring by the segregation of the genes B and P (Hermsen and Verdenius 1973; Jacobsen and Sopory 1978). The results indicated that the regenerated diploid plants descended from reduced microspores although only a few plants revealed 12 chromosomes.

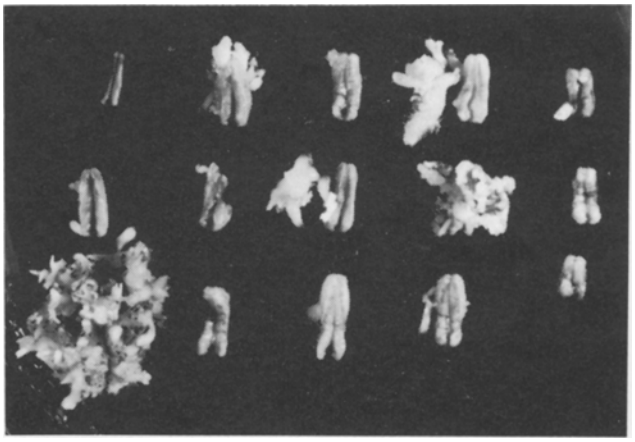

Fig. 1. Anther culture of the high responsive dihaploid potato clone H 79.1038/27, five weeks after plating on the induction medium

At that time the number of clones which could be regenerated through anther culture in reasonable quantities was limited. Figure 1 represents a good regeneration response from anthers: nearly all of them developed macroscopic structures. Clones possessing resistances, however, did not respond in sufficient quantities to our routine regeneration procedure. A total of more than 20 dihaploids were intensively tested (to classify a clone as non-responsive, 300-500 plated anthers must have failed to develop macroscopic structures). Amongst the tested

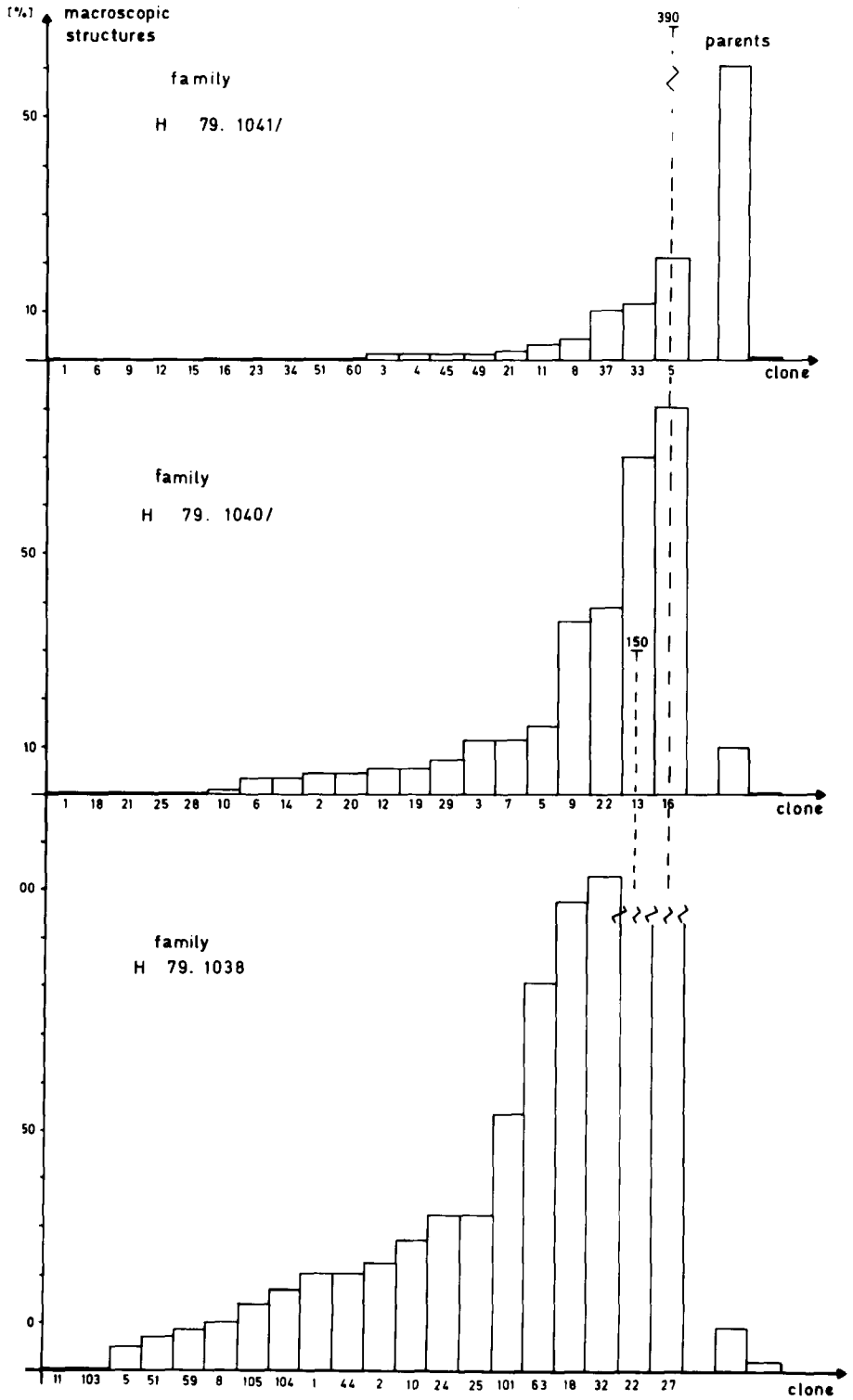

Fig. 2. Macroscopic structure formation (in % of the number of plated anthers) from 20 different clones of three different F_1 hybrid families. One parent possessed a good regeneration capacity; the other a resistance

resistant clones only clone H 78.2020/26 with wPCN resistance and clone H^2 59 with field resistance to PLRV and PVY developed a few macroscopic structures.

1 Regeneration Capacity

Only a few genotypes showed a frequency of macroscopic structure formation over 10%, which is a prerequisite for the production of plants in reasonable numbers for applied purposes. Hence, two approaches can be adopted: 1. Modification of the culture conditions, 2. modification of the genetic background of the plants. As the first approach is purely empirical, we followed the second. In Figure 2 data are summarized which demonstrate the behaviour of three F_1 hybrid families, each of which was combined from a clone having given good results in anther culture and a non-responding, but valuable second clone. The F_1 family was firstly screened for the presence of PVX, PVY or wPCN resistance, and then the resistant ones served as anther donor material. In Figure 2 only those clones are represented where more than 300 anthers have been plated. The response of such hybrids varied greatly: family H 79.1041, a hybrid between the poorly, but highly PVY and PLRV field resistant clone $H^2$473 and the good responding clone $H^3$703, showed few good regenerating clones. (Data for clone H^3 703: 1,441 plants were regenerated from 11,185 macroscopic structures having arisen from 17,910 plated anthers). The segregation of the other two representative families in Figure 2 was more distinct: family H 79.1040 is a cross between clone H78.01/110, which gave a good response in anther culture, and clone H^3 704, with field resistance to viruses. Family H 79.1038 is combined from clone H 78.01/110 and H^2 59. From this family some clones produced very high amounts of macroscopic structures, up to nearly 400% per plated anthers (H 79.1038/27). However, the majority of these structures developed into callus, therefore the number of regenerating plantlets is still relatively small (Table 1). It is likely that most calluses will show morphogenesis after prolonged culture on the regeneration medium II.

The behaviour of such F_1 hybrid families indicated that the capability of producing macroscopic structures from microspores is under a genetic control. It is, however, too early to state anything about the mode of inheritance. According to this point it could, nevertheless, be demonstrated, that the segregation may be

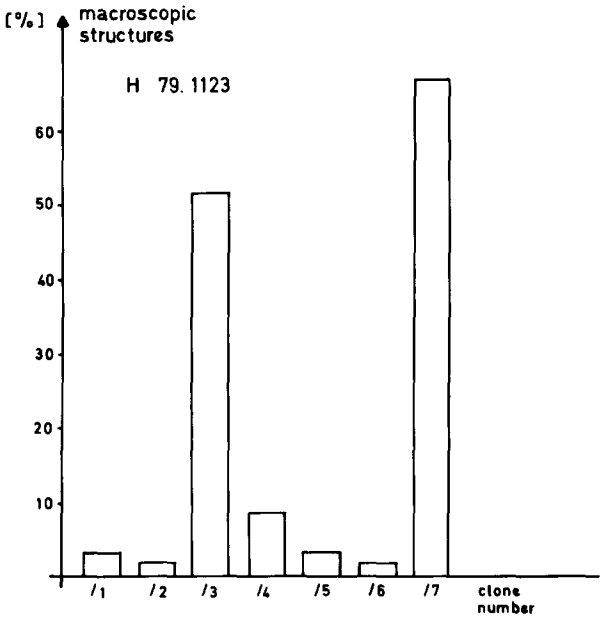

Fig. 3. Macroscopic structure formation from a F_1 hybrid family; one parent of which had passed anther culture and thus probably possessed the regeneration capacity in the homozygous condition

Table 1. Data for some clones regenerated via anther culture (H = dihaploid; A = anther derived 2x clones)

Anther donor clone	Resistant to	Number of		Number of clones regenerated				
		Anthers plated	Macroscopic structures	Total	% of anthers	functional 1x	2x	4x
H 78.01/10	PLRV	2,452	1,620	517	21	63	260	10
H 78.01/27	PLRV	3,765	2,880	2,564	68	401	1,091	30
H 78.2020/26	wPCN	1,303	89	28	7	–	28	–
H 78.2022/14	wPCN	6,031	492	2	0.03	–	2	–
H 79.1038/18	PVY	831	1,009	48	6	12	28	3
H 79.1038/27	PVY	833	5,049	232	28	1	64	11
H 79.1041/37	PVY	1,324	172	10	1	1	8	1
H 79.1045/11	PVX	324	2	2	1	–	2	–
A 79.01/41	PLRV ?	15	69	65	433	–	54	1
A 79.01/68	PLRV ?	75	163	48	64	1	42	1
A 79.01/77	PLRV ?	45	136	26	58	–	18	–

more distinct into two different classes (Fig. 3). Here the anther donor clone H 79.1123 is a cross between the clone H^3 692 with no macroscopic structure development at all, and the clone A 78.5026, a spontaneous diploid clone of microspore origin which should possess the regeneration capacity in the homozygous condition.

Besides this probably inheritable tissue culture ability, the passage via anther culture, has a further effect on the genome. It is possible that different clones possess different amounts of sublethal gene combinations. The alleles are present in the heterozygous condition in the diploid plant but become active in the monohaploid or homozygous diploid state. This implies that during anther culture there exists a strong selection for genotypes free of lethal genes. Indeed, plants derived from microspores were rather vigorous, and a high proportion of them did not express inbreeding depression. This view is further strengthened by the high frequency of androgenesis observed in anthers taken from A_1 plants (Table 1; clones A 79.01/41; A 79.01/68 and A 79.01/77); during the first anther culture cycle most deleterious genes were lost.

The strong influence of the genotype was also underlined by the rather different frequencies of functional monohaploid potatoes descending from different donor clones. Their frequency ranged from zero (e.g. A 79.01/41) to nearly 40% (clone H 78.01/27; Table 1). Furthermore, we found that during the transfer of androgenetic macroscopic structures from the induction to regeneration medium I an average of 70% died. The survival rate was hardly influenced by different regeneration media: a specific genotype will grow on nearly all medium variations, while the others do not grow on any medium (Fig. 4), thus the medium cannot be the critical factor. But again sublethal alleles may be responsible: macroscopic structures being in close contact with each other and with the anther tissue are able to survive because of cross feeding. On the isolation and transfer to the regeneration medium I with the decreased sucrose level, the lethal ones will die. As clone H 78.01/27 demonstrates, there exists also unselected clones where nearly all macroscopic structures will regenerate; probably only a few sublethal alleles are present in this genotype.

The question is, how far the lethal ones are auxotrophic mutants, which by supplementation with maximal media can survive. Experiments along this line are in progress.

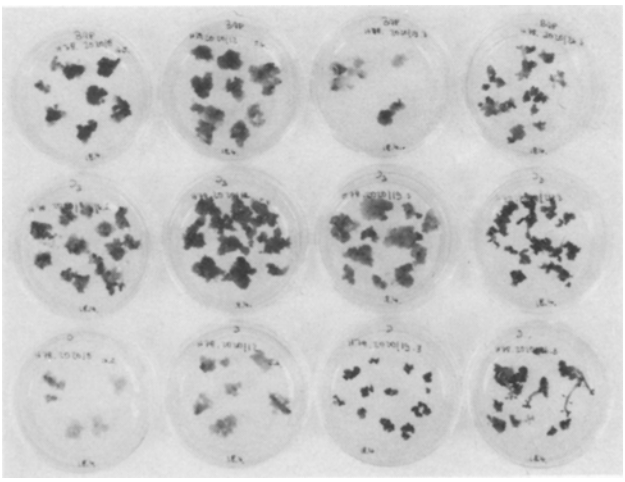

Fig. 4. Anther derived potato clones (each callus represents a different genotype) on an aliquot of three different media combinations. Despite the phytohormone combination and concentration only regenerants from clone H 78.2020/26 show morphogenesis (right row)

2 *Incorporation of Resistances*

In Table 1 characteristic data are summarized which have been obtained after anther culture from plants possessing resistance in the heterozygous condition. The detailed behaviour of two families regarding the inheritance of resistance after a monohaploid step is outlined in Table 2 for wPCN and in Table 3 for PVY.

2.1 Nematode Resistance

From the highly wPCN resistant clone H 78.2020/26 with an P_f/P_i value of 0.06, 15 clones were intensively

Table 2. Segregation of the resistance to wPCN in anther derived clones from a highly resistant wPCN dihaploid anther donor clone

	No of newly formed cysts		P_f/P_i value for	
Anther donor clone	Per bio vessel	Pot	Bio vessel	Pot
H 78.2020/26	o[a]	–	0.06[a]	–
Anther derived clones				
A 80.2200/2	8	12	0.43	0.61
/3	14	10	0.67	0.34
/4	4	4	0.22	0.26
/6	9	14	0.60	0.59
/12	8	10	0.31	0.36
/15	0	0	0	0
/16	2–[b]	13	0.10	0.78
/18	–	23	–	0.90
/19	–	13	–	0.78
/21	–	14	–	0.80
/22	0	16	0	0.76
/23	0–	5	0	0.17
/24	0–	9	0	0.45
/25	1	0	0.06	0
/26	10	7	0.51	0.41

[a] Average value from three repetitions
[b] Poor root development

Table 3. Segregation of the quantitatively inherited PVY field resistance after anther culture.

	PVY inoculation to	No. of clones	
		Susceptible	Resistant
F_1 family H 79.1041	No. of seedlings 1,299	600	699
F_1 anther donor clone	No.[a] of A_1 clones tested		
H 79.1041/4	1	1	0
/8	6	1	5
/11	1	1	0
/14	4	4	0
/18	11	3	8
/25	1	1	0
/37	6	2	4
/49	2	0	2
/64	1	0	1

[a] At the average 10 plants were inoculated per clone

analyzed. All clones obtained through anther culture showed a good level of resistance, ranging from a P_f/P_i value of 0.06, equivalent to the donor clone, to 0.90 (A 80.2200/18). (Even this clone will reduce the cyst number in the field). This means that the quantitatively inherited resistance to wPCN, extracted from *S. vernei*, was not lost during the reduction of the ploidy level from 2x to 1x. Quite probably the resistance is now present in the homozygous condition, which means that a polygenically inherited resistance can also be transferred to the next generation with a very high efficiency.

2.2 Virus Resistance

In the case of PVY 46 microspore derived clones from two different families and a total of 15 different anther donor clones were analyzed after mechanical PVY inoculation. The data for family H 79.1041 are given in Table 3. Although in the case of PVY the numbers are too small to run statistical analysis, it can be seen that in some cases, e.g. in the anther donor clone H 79.1041/14, all androgenetic clones showed less resistance than the parental clone (or that the parent was an escape during the rapid screening for PVY resistance); in other cases most clones tested showed resistance, while some segregated. To identify more precisely different levels of resistance the plants were inoculated with different virus concentrations (dilutions of the crude sap of 1:10; 1:100 and 1:1,000). It was never found that the high concentration caused virus infection, while the low concentration did not.

The resistance to PVY and PLRV was tested additionally in a field test. Tubers harvested in the greenhouse from the first androgenetic plants with a low field resistance to PVY and PLRV were planted in the field in Köln under high virus pressure. From 1,957 tested clones in 1979, an aliquot of 230 was harvested and regrown in 1980. In the second year they were screened for natural field resistance to PVY and PLRV. The results are given in Table 4 (H^3 703). It can be seen that from this clone a higher proportion of anther derived clones was susceptible, than in the clones, where a higher level of resistance was present in the anther donor plants. Figure 5 demonstrates the appearance of this regrown field plot.

Table 4 summarizes results obtained for the segregation of resistances in androgenetic clones. From a hybrid with extreme PVX resistance, H 79.1045, only from one clone could two androgenetic clones be regenerated. One of these was resistant when grafted on a PVX infected tomato, the other one was also resistant after mechanical inoculation but became infected after grafting. This segregation does not yet tell anything about the proportion of extreme resistant plants. It should be possible, however, to determine by the segregation pattern the

Table 4. Summary of the resistance in 2x regenerants after androgenesis

Anther donor clone	Resistant to type	A_1 clones tested		
		Total	Susceptible	resistant
H^3 703	PLRV low PVY field res.	230	146	84
H 78.2020	wPCN highly res.	15	1	14
H 79.1038	PVY field res.	12[a]	6[a]	6
H 79.1041	PVY field res.	34[a]	14[a]	20
H 79.1045	PVX extrem res.	2	1	1

[a] Including one monohaploid clone each

Fig. 5. Anther derived 2x plants (two plants per clone) regrown in the Cologne field for the second year. Virus infected and visually healthy plants can be distinguished

number of genes responsible for the resistance, as soon as more A clones are screened.

As homozygous dihaploids may also be of interest for true seed production, we screened some clones for self-compatibility. Amongst the 600 plants tested 13 self-compatible clones were found. In combination with resistances such clones can be propagated like a self-compatible crop and may also serve as starting material for hybrid seed production. The final return to the tetraploid level – if necessary – can be undertaken either by meiotic doubling or somatic hybridization. Experiments along this line using protoplasts are in progress (Schieder, Uhrig and Wenzel, unpublished). For these experiments a new selection system for the identification of *S. tuberosum* + *S. tuberosum* hybrids is under trial (Uhrig 1981).

Discussion

One of the strongest obstacles against efficient resistance breeding in potato is its tetraploid nature. In our breeding scheme (Wenzel et al. 1979) we reduced through parthenogenesis the ploidy level to 2x, where we found strong influences of the female. This was also true for the second step, where the parthenogenetic method works as well (van Breukelen et al. 1975). The second parthenogenetic step is, however, more laborious as no natural selection against 2x hybrids operates. As a consequence many more seeds and seedlings have to be screened for their ploidy level. Further, there is no simple possibility of identifying amongst dihaploid *S. tuberosum* × *S. phureja* hybrids the spontaneous doubled monohaploid *S. tuberosum* clones, which are present in quite high quantities (Hermsen pers. communication) in addition to the few monohaploids (5 from 260,000 seeds, Wenzel 1979; 0.1% for a specific Gineke dihaploid, van Breukelen et al. 1975).

Therefore, we concentrated on androgenetic monohaploid plant production. In experiments along this line two results were reported here: 1. The regeneration capacity of microspores was dependent upon the genotype and could be transferred via sexual combination breeding. 2. From hybrids carrying resistances in the heterozygous condition homozygous resistant clones could be produced via anther culture despite the mode of inheritance.

According to the first point we tend to explain the regeneration capacity with a different specifity of different genotypes against exogenous influences by the media, most probably by the phytohormone concentrations. This external influence is still not well understood, but both the exo- and endogenous phytohormone concentration influence the regeneration potential. Genotypes which are not too sensitive and which do not require highly balanced phytohormone levels are the ones which react more frequently. Others are just irreversibly destroyed by the influence of the medium. Sensitivity and insensitivity may be under such genetic control as reported for red clover (Keyes et al. 1980). Simon and Peloquin (1977) discuss a dominant inheritance for the amount of callus formation from potato anther tissue. There is surely not just one step where this system acts. As it can be seen, e.g. in the anther donor clones H 78.2022/14 and H. 79.1038/27, the induction of macroscopic structures can be triggered with a high frequency, while the formation of plants is limited. Hence, we think that the chance for a successful regeneration will be greater by bringing tissue culture tolerance into genotypes, than by finding for each genotype a specific medium. This does not mean that there exist genotypes with no tissue culture ability at all; probably for each type a specific medium can be successfully composed. The results reveal that via sexual combination the regeneration capacity can be incorported into clones on a broad basis, enabling a less restricted use of tissue culture techniques.

The high number of field resistant clones which could be regenerated from anther donor plants heterozygous for this character can be explained either by the number of alleles involved, which is still high enough to cause resistance after ploidy level reduction, or with their origin from unreduced FDR microspores. The origin of plants from unreduced microspores may also explain the observation that some anther derived clones showed segregation after selfing, especially when checked for the anthocyanin and flavonol pigment patterns (Wenzel et al.

1981). As here only slight differences were observed, these plants may also result from SDR microspores, where cross-overs had occurred and resulted in parts of the genome being heterozygous. In our opinion the origin of unreduced microspores can only partially explain the high numbers of resistant or heterozygous clones, because the phenotypic segregation, e.g. of tuber skin and shape, is too high to support their origin from unreduced microspores. Final proof, however, may come only from the culture of isolated microspores (Sopory 1977) separated by physical means into reduced and unreduced ones, much like separation of viable from unviable ones is possible (Wenzel et al. 1975; Wernicke et al. 1978).

There are several different findings in other anther derived populations (de Paepe et al. 1981; Picard 1980), where anther derived doubled haploid lines showed again some segregation after a sexual cycle; perhaps complete homozygosity can never be kept in a genome because of somatic mutations. Such small changes do become evident, however, only when complete homozygosity is achieved, as in doubled haploids. This homozygosity is surely much more complete than the homozygosity present in an inbred line of a selfcompatible crop.

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