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ANTHER CULTURE IN TUBEROUS SOLANUMS

I. Callus Formation and Root Differentiation*

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Abstract

Anther culture has been carried out with four tuberous diploid *Solanum* species (2n=24), *S. berthaultii*, *S. chacoense*, *S. jamesii* and *S. phureja* including two clones, in oder to find out conditions suitable for inducing haploid plantlets from the pollen, in all combinations of various anther stages, basal media and growth substances.

Calluses induced from the cultured anthers were provisionally classified into three types according to their probable origin; calluses originating from inside the pollen sacs, calluses proliferating from the cut ends of the filaments, including ones from the tissues connecting the pollen sacs, and the anther wall dedifferentiating into callus. The first-mentioned calluses, some of which may be expected to be of pollen origin, were induced 5~6 weeks after inoculation, provided that anthers were selected at the early uninucleate stage of microspore development, that they were incubated on NITSCH & NITSCH's basal medium containing mineral salts in a relatively low concentration, and that the medium was supplemented with 1.0 mg/l of IAA and each of 1.0, 5.0 and 10.0 mg/l of Kinetin in combination. Under such conditions *S. phureja* did produce calluses of this type, with the mean frequency of approximately 3% of the anthers involved, but the other species all failed to respond in any case. Calluses of the other types were frequently observed, no matter what species, anther stages and culture media were used. Some of the calluses produced roots when transplanted into the same basal medium containing 1.0 mg/l of IAA combined with each of 1.0 and 5.0 mg/l of kinetin. The roots, however, failed to develop and died some time later, before checking their chromosome numbers. No shoot differentiation was eventually found in any of the instances.

Production of haploid plants from the pollen by means of anther culture was first demonstrated by GUHA and MAHESHWARI [4,5] with *Datura innoxia*, and then a considerable success in this way has been obtained for *Nicotiana* [2,13,18,21] and *Oryza* [14~16]. This subject, since, has become of special interest to plant breeders, and a number of attempts have been made, with or without success, to adapt this method to certain other genera of plants [3,6,7,9,10,20,24]. Recently, success with tuberous *Solanum* has also been reported in a few species[8].

In tuberous *Solanum*, monoploids are indispensable as a useful means not only for raising homozygous clones but also for deciding exactly a basic chromosome number of the genus. In this respect, however, nothing definite has been so far known.

The present investigation was thus undertaken, the aims being to induce monoploids in certain diploid species of the genus by culturing their anthers *in vitro*, and to approach the above problems to solve using monoploids obtained. In this paper an account is given of the effects of anther stages, basal media and growth substances both on the callus formation from anthers and on the root differentiation from the resulting calluses, and also of the response of the *Solanum* species used to anther culture.

Materials and Methods

The following four diploid *Solanum* species (2n=24) including five clones were used in this investigation.

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** Laboratory of Plant Breeding.

S. berthaultii HAWKES, PI 218215.
S. chacoense BITT., H82.
S. jamesii TORR., C-381 x C-383.
S. phureja JUZ. *et* BUK., (formerly *S. rybinii* JUZ. *et* BUK.) Clone 1, PI 197761; Clone 2, PI 197762.

The materials with the PI numbers had been supplied by Dr. P. R. ROWE from the Inter-regional Potato Introduction Station, U. S. A., and *S. chacoense* and *S. jamesii* by Dr. J. G. HAWKES from the Botany Department, University of Birmingham, England and by Dr. K. M. GRAHAM from the Research Station, Canada Department of Agriculture, Canada, respectively.

Floral buds for culture were selected by cytological checking in acetocarmine anther squashes. They were washed in detergent, surface-sterilized by dipping in 70% ethanol for a few seconds, followed by immersing in 1% sodium hypochlorite solution for 10 minutes and then rinsed several times with sterile water. The anthers were excised aseptically and planted in 21 x 120mm test tubes, each containing 10ml of the medium, with five anthers per tube. The cultures were all incubated in darkness at temperatures around 27°C.

The basal media used were prepared on the formulae described by NITSCH (1951)[17], MURASHIGE & SKOOG (1962)[12], LINSMAIER & SKOOG (1965)[11], BLAYDES (1966)[1] and NITSCH & NITSCH (1969),[19] though evenly with the sucrose concentration of 3%, and each supplemented with growth regulating substances; indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and kinetin. These supplements were each manipulated in the concentrations of 0.1, 1.0, 5.0 and 10.0 mg/l, and used both alone and in such combination as each treatment consists of one auxin and one cytokinin. In all cases the media were adjusted to pH 5.8~6.0 and solidified with 0.8% agar. Assessment of the results was made by counting the number of the anthers from which calluses were induced and by assorting these calluses according to their probable origin, as described in the next section.

Results and Discussion

Callus Formation

Favourable anther stage

For assessing the anther stage suitable for culture, floral bud lengths were measured in relation to the microsporogenetic stages. The results obtained with *S. phureja* are given in Table 1. Anthers at four stages were selected for culture; early tetrad, late tetrad, early uninucleate and late uninucleate stages. After 4~8 weeks in culture, calluses occurred from some of the anthers, with such frequencies as shown in Table 2. The calluses were classified into three types according to their probable origin, that is as follows (Fig. 1):

Callus A — derived from inside the pollen sacs.
Callus B — proliferated mostly from the cut ends of the filaments and occasionally from the tissues connecting the pollen sacs.

Table 1. Anther stages in relation to the floral bud lengths in *S. phureja*.

Floral bud length (mm)	Anther stage
2.3-2.6	Archesporial
2.8-3.0	Mature pollen mother cells prior to meiosis
3.3-3.4	Early prophase
3.6-3.8	Diakinesis to second anaphase
3.9-4.0	Early tetrad
4.1-4.2	Late tetrad
4.4-4.6	Early uninucleate
4.8-5.2	Late uninucleate
6.5-7.5	Binucleate

Table 2. Callus formation from the anthers cultured at different stages of microspore development in *S. phureja*.

Stage	No. of anthers cultured	Frequency of anthers forming: Callus A* No. (%)	Callus B* No. (%)	Callus C* No. (%)	Total No. (%)
Early tetrad	80	0	3 (3.8)	3 (3.8)	6 (7.6)
Late tetrad	76	0	5 (6.6)	9 (11.8)	14 (18.4)
Early uninucleate	80	2 (2.5)	2 (2.5)	6 (7.5)	10 (12.5)
Late uninucleate	80	0	4 (5.0)	11 (13.8)	15 (18.8)

*For explanaton see text.

Callus C——the anther wall dedifferentiating into callus.

Of these, calluses of the first type may be expected as a matter of course to include those of pollen origin, although they must occasionally be contaminated with calluses arising from some somatic tissues inside the anther wall such as tapetum, middle layer or connective tissue. Such calluses were obtained, with a frequency of 2.5% of the anthers involved, within a period 5 to 6 weeks after inoculation, only when the anthers that contained microspores being in the uninucleate state were cultured. Calluses B and C were found in every instance, their occurrence being at frequencies of 2.5~6.6% and 3.8~13.8% respectively, and there was not seen a definite relationship between callus formation and anther stage.

On this subject, it has been so far known that microspores destined for embryoidal formation are within the limits ranging from tetrad to late uninucleate stages. In *Nicotiana tabacum*, anthers containing uninucleate grains at the time of culture have been found to be available for giving rise to embryoids of pollen origin[18,19], and, furthermore, the older ones in this range have been pointed out to be more successful[22]. The same is true for the present materials, desirable calluses being obtained from the anthers at early uninucleate stage, even though their entity is not yet ascertained.

Effect of the culture medium compositions

a). Basal media

A comparison was made between the five basal media mentioned above for their effect on callus induction. These media each were added with IAA 1.0 mg/l and kinetin 5.0 mg/l and anthers of *S. phureja* were incubated on them. Callus A was found only in the case where NITSCH & NITSCH's medium was used, out of 80 anthers examined 4 (5%) giving rise to calluses of this type. Callus B occurred in every case, though their frequencies varying widely with different media. In NITSCH & NITSCH's medium 5.4% of the anthers inoculated were of such a type, whereas only 0.6~1.8% were counted in the other media. Similarly Callus C was seen in every case and its frequency did not appreciably differ among the media, showing a range 6.5 to 8.4%.

Thus, NITSCH & NITSCH's medium was the most favourable one of the media tried to induction of calluses, especially of type A, from the anthers. Comparing these media with one another for their mineral content, NITSCH & NITSCH's is, though similar to BLAYDES', more than twice as high as NITSCH's and approximately one-half lower than both MURASHIGE & SKOOG's and LINSMAIER & SKOOG's. Such differences among the media seem to affect the incidence of callus formation from the anthers cultured on them. The minerals composing NITSCH & NITSCH's medium would be probably in a favourable concentration to anther cultures of the present materials.

Table 3. Effects of growth regulating substances on the callus induction from the cultured anthers of *S. phureja*.

Growth sub. added(mg/ℓ)		No. of anthers cultured	Frequency of anthers forming					
			Callus A*		Callus B*		Callus C*	
IAA	kinetin		No.	(%)	No.	(%)	No.	(%)
10.0	10.0	80	0		0		0	
10.0	5.0	75	0		0		0	
10.0	1.0	80	0		0		0	
10.0	0.1	80	0		1	(1.3)	0	
5.0	10.0	70	0		5	(7.1)	15	(21.4)
5.0	5.0	70	0		9	(12.9)	11	(15.7)
5.0	1.0	80	0		5	(6.3)	8	(10.0)
5.0	0.1	80	0		3	(3.8)	4	(5.0)
1.0	10.0	70	2	(2.9)	4	(5.7)	17	(24.3)
1.0	5.0	80	4	(5.0)	11	(13.8)	7	(8.8)
1.0	1.0	75	1	(1.3)	12	(16.0)	14	(18.7)
1.0	0.1	80	0		3	(3.8)	3	(3.8)
0.1	10.0	80	0		5	(6.3)	3	(3.8)
0.1	5.0	75	0		11	(14.7)	10	(13.3)
0.1	1.0	70	0		5	(7.1)	9	(12.9)
0.1	0.1	80	0		0		2	(2.5)
0	0	80	0		0		0	
Total		1305	7	(0.5)	74	(5.7)	105	(8.0)

*For explanation see text.

b). Growth regulating substances

Effects of growth substances on callus formation were tested by culturing *S. phureja* anthers on NITSCH & NITSCH's medium added with IAA, NAA, 2, 4-D and kinetin in various concentrations ranging 0.1 to 10.0 mg/l, these substances being used both alone and in com bination of one auxin and one kinetin.

When the substances each were used alone, no formation of Callus A was observed in any case. Calluses of types B and C, however, occurred when the auxins concerned were provided in concentrations of 1.0 and 5.0 mg/l, and their frequencies were confined within the range 0.2 to 2.6% of the anthers cultured. Kinetin and 2, 4-D did not exert any influence on inducing calluses from anthers.

Callus formation, on the other hand, appeared to be enhanced by adding the auxins in combination with kinetin to the medium. When NAA was combined with kinetin Callus B arose in 4.8% of the anthers and Callus C in 6.3%, and when 2, 4-D was combined with kinetin 5.8% and 11.2% were counted respectively. These frequencies, thus, are apparently high as compared with those in the cases where the substances were used alone. Callus A, however, was not obseved in any of these cases. It was found only when IAA was combined with kinetin as noted in Table 3. In this table there are given the frequencies of the anthers from which calluses of the three types were found on the media added with IAA combined with kinetin in various concentrations. It is seen from the table that Callus A was induced in 7 out of a total of 1305 anthers cultured and it occurred only in the media containing 1.0 mg/l of IAA and each of 1.0, 5.0 and 10.0 mg/l of kinetin in combination, with a mean frequency of about 3% of the anthers involved. On these media Calluses B and C were also seen more frequently than on the others.

Of special interest, here, is that callus induc tion from inside the anthers depends largely on the presence of IAA combined with kinetin at a certain level in their concentration. Neither the presence of IAA at either a more or less level than 1 ppm nor that of kinetin at other levels than 1~10 ppm appears to be responsible for triggering the formation of callus of this type. Such a situation, however, may be applicable only to tuberous *Solanums*. In *Nicotiana*, it has been pointed out that any growth substances are not essential for inducing embryoids from the pollen[18,19], and the same is true in *Lycopersicon*[20]. In *Solanum*, on the contrary, pollen-calluses have been successfully obtained on the medium supplemented with NAA, 2, 4-D and kinetin in concentration as high as 2 ppm each[6,8], and also in *Paeonia* the induction of multicellular grains from the pollen has been known to be favoured by addition of either NAA or 2, 4-D 0.1 mg/l and kinetin 5.0 mg/l [23]. Such being the case, the effects of growth substances on the callus formation from the pollen are considerably different from one species to another. This may be interpreted as a result of species differing from one another in their requirement to exogenous hormones due probably to endogenous hormone levels varying in different species.

Table 4. Callus formation from anther cultures of the different *Solanuu* species used.

Material	No. of anthers cultured	Frequency of anthers forming Callus A* No. (%)	Callus B* No. (%)	Callus C* No. (%)
S. berthaultii	135	1 (*0.7*)	13 (*9.6*)	14 (*10.4*)
S. chacoense	120	0	11 (*9.6*)	16 (*13.3*)
S. jamesii	185	0	0	66 (*35.7*)
S. phureja Clone 1	120	4 (*3.3*)	11 (*9.2*)	18 (*15.0*)
S. phureja Clone 2	105	3 (*2.9*)	16 (*15.2*)	20 (*19.0*)

*For explanation see text.

Response of the materials used to anther culture

Anthers from the four species mentioned above, including five clones, were cultured on NITSCH & NITSCH's medium added with 1.0 mg/l of IAA and each of 1.0, 5.0 and 10.0 mg/l of kinetin in combination, and the results obtained are presented in Table 4. Callus A was obtained in both *S. berthaultii* and *S. phureja*, its frequency being 0.7% of the anthers in the former and 3.3% and 2.9% in two clones of the latter.

Callus B was found in all the species used, except for *S. jamesii*, with frequencies ranging 9.2 to 15.2%. Calluses formed in *S. jamesii* all were of type C and more than one-thirds of the anthers produced such ones.

It has been pointed out in *Nicotiana* and *Solanum* that species vary markedly in their ability to form callus from the anthers [8,18,23]. The present materials were also variable in their response to callus formation. The reason for this is not obvious at present, though, as mentioned already, it may be attributable to inherent hormonal requirements varying in different species.

Root Differentiaton

When calluses of the types A and B fully developed, they were transferred to the media supplemented with IAA and kinetin at ratios of 1:5, 1:1 and 5:1, each in concentration of mg/l, and examined for organ differentiation from them.

Root formation occurred from some of these calluses for a period ranging 4 to 6 weeks after transplanting. Table 5 shows the frequency of calluses from which roots differentiated. Rooting from Callus A was found only on the medium with a combination of IAA 1.0 mg/l and kinetin 1.0 mg/l, and Callus B produced roots on the medium containing each of 1.0 and 5.0 mg/l of IAA in combination with kinetin 1.0 mg/l. In both cases, the presence of higher auxin and lower kinetin levels appeared to be favourable to induce roots from the calluses. Two types were discernible in rooting from the callus; one showing thick and robust roots growing vigourously and the other showing slender and hair-like roots emerging from the surface of callus (Fig.2). These roots continued to grow, but, for some unknown reason, they degenerated a few weeks after their emergence, thus their chromosome numbers remaining undecided. No shoots were eventually formed in any of the cultures, even after long-continued sub-culture.

Table 5. Root differentiation from the anther-derived calluses in *S. phureja*.

Growth sub. added(mg/ℓ)		Callus A*		Callus B*	
IAA	Kinetin	No. of calluses sub-cult.	Rooting	No. of callsues sub-cult.	Rooting
5.0	1.0	2	0	10	1
1.0	1.0	3	1	10	2
1.0	5.0	2	0	10	0

*For explanation see text.

From the results obtained throughout this work, the following main conclusions will be drawn in respect of the response of the cultured anthers to growth substances added to the medium. The first is that among the auxins used IAA appears to be most effective not only in inducing calluses from the anthers, especially from inside their pollen sacs, but also in giving rise to roots from the callus, and that its effect is likely to be enhanced when combined with kinotin. The second is that whether the cultures are diverted their growth into dedifferentiation or redifferentiation seems to be largely dependent on a relative concentration of IAA to kinetin, the former tending to be favoured in the presence of IAA at a lower level and the latter being contrary to this.

This view, however, is not always agreeable with the results obtained with other genera by some earlier workers stated already. It is not explainable at present whether this is due to a nature inherent in the present *Solanum* materials or to other factors, and hence there is a clear need for further investigations.

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バレイショ近縁種における葯培養

I. カルスの形成と根の分化

松 林 元 一

要 約

花粉起源の半数体を育成するために，バレイショの2倍性近縁種(2n=24)，*S. berthaultii*, *S. chacoense* *S. jamesii* び *S. phureja*(2栄養系)を用いて葯培養を行ない，葯からのカルス形成とカルスからの根の分化に対する置床葯の発育期，基本培地の組成ならびに生長物質添加量の影響を供試材料別に比較検討した。培地は NITSCH(1951), MURASHIGE & SKOOG(1962), LINSMAIER & SKOOG(1965), BLAYDES(1966)および NITSCH & NITSCH (1969) の処方を基本とし，これにIAA, NAA, 2, 4-D および kinetin を 0.1, 1.0, 5.0および10.0*mg*/*l* の濃度で単独または混合添加した。

供試材料や培養条件のいかんをとわず，葯内容からの直接の胚状体形成はみられず，いずれの場合も培養による初期変化としては，葯内部からのカルス形成 (A)，花糸切断面でのカルス形成 (B)，ならびに葯壁のカルス化 (C) のみであった。これらのうち，花粉起源が期待されるAカルスは，置床5～6週後，つぎの条件が整ったとき，置床葯数の約3%の頻度をもってその誘導が認められた。すなわち，供試材料別では *S. phureja* を，また葯の発育期別では1核初期の葯を用いた場合，さらに培地組成別では無機塩類濃度の比較的低い NITSCH & NITSCH の基本培地にIAA1.0*mg*/*l* と kinetin 1.0, 5.0 および 10.0*mg*/*l* を混合添加した場合がこれである。 B カルスは，IAA 10.0*mg*/*l* 区を除いては，kinetin の量とは関係なく，置床葯数の1.3～16.0%の頻度をもってその形成がみられた。Cカルスの形成もほぼこれと類似の傾向を示した。いずれの場合も NAAと2, 4-Dの添加区からは，それらの単用もしくは kinetin との併用をとわず，IAAと kinetin との併用にまさったカルス形成率はえられていない。一方，カルスからの根の分化は，kinetin 1.0*mg*/*l* に等量の IAA を併用した NITSCH & NITSCH の培地にカルスを移植したとき，その4～6週後に，A・B両カルスで，また IAA の量比をあげて5.0*mg*/*l* とした場合にもBカルスでそれぞれ認められた。発根には2様式がみられ，Aカルスは太く逞しい根系を，B カルスはその表面から根毛様の細根を分化した。しかしこれらの根はその後まもなく褐変枯死したので，それらの起源を確認するにいたっていない。茎葉の分化はいずれの場合にも認めることができなかった。

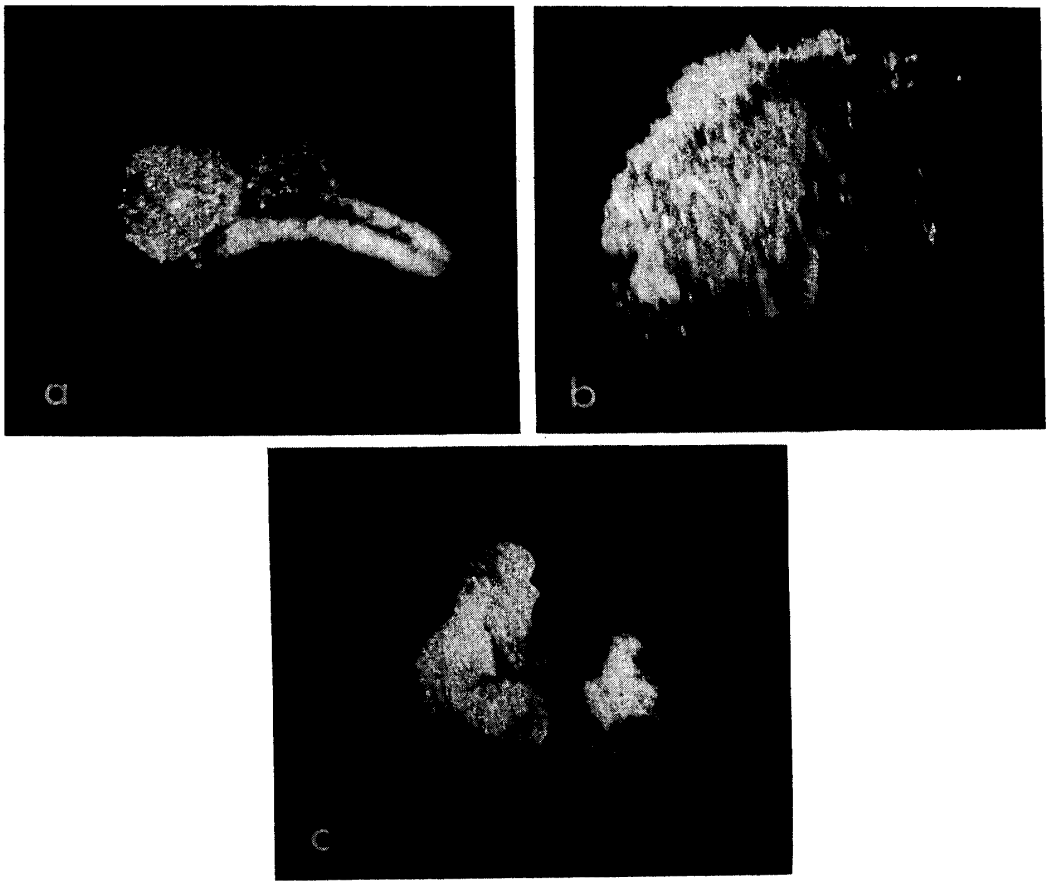


Fig. 1. Three types of callus formation from the cultured anthers of *S. phureja*. a), b) and c) show the callus of types A, B and C, respectively. For explanation see text.

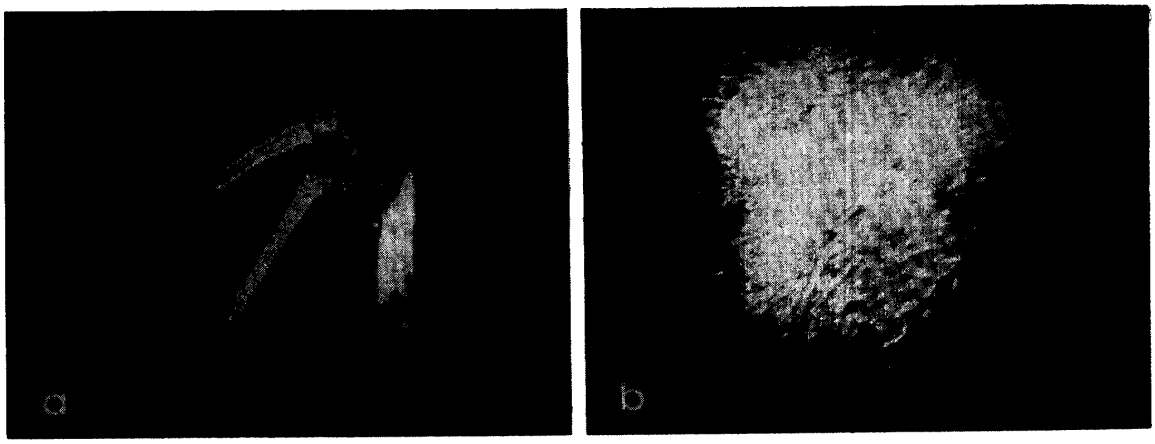


Fig. 2. Root formation from the sub-cultured calluses in *S. phureja*, showing two types of rooting. a). Thick and robust roots growing vigorously from Callus A. b). Slender hair-like roots emerging from the surface of Callus B.