Collaborative Program AVRDC/CIRAD

Set Up of Techniques for:
- Onion Breeding and
- Garlic Dissemination

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- PUBLICATION **; Embryogenic Cell Suspension Cultures of Garlic (Allium sativum L.) As Method For Mass Propagation and Potential Material For Genetic Improvement.** ***2005, "Acta Horticulturae 688, p65-74"***
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- POSTER **; Production de masse de plants sains d'ail par embryogénèse somatique. 2005. Journées Horticoles CIRAD.** L. Fereol; V. Chovelon; S. Causse, M.L. Kalumvueziko and R. Kahane ; AVRDC World Vegetable Center, P.O. Box 42, Shanhua, Tainan, 741 Taiwan R.O.C.

- PROJECT PUBLI **; Potentialities of Garlic somatic embryogenesis for elimination of OYDV and LYSV.** (in progress)

- PROJECT BOOK SECTION **; : Tissue culture and its role in plant biotechnology.** (in progress); AVRDC World Vegetable Center, P.O. Box 42, Shanhua, Tainan, 741 Taiwan R.O.C.

OBTENTION OF DOUBLE HAPLOID OF ONION IN THE CONTEXT OF ONION BREEDING

1 Background information

AVRDC's breeding program of onion focuses its objectives on increasing yield, improving storability, and eliminating diseases. Concerning disease resistance, particularly against Stemphyllium vesicarium, AVRDC is incorporating such traits into short day onion cultivars. Allium fistulosum Stearn (a wild relative of onion) is known to be resistant to Stemphylium leaf blight (caused by Stemphylium vesicatoria) (Pathak et al., 2001). This wild onion relative is sexually crossable with common onion (*A. cepa* L.), providing partially fertile offspring (Pathak et al., 2001). The strategy of onion breeding can help to transfer the resistance gene to stemphyllium and over coming the sexual barrier between the two species. But onion breeding is a slow process primarily due to the biennial nature of this outcrossing species. Development of an onion inbred line may require more than 12 years, when the traits of interest are transferred from interspecific crosses. Doubled haploid (DH) techniques can shorten the time required to develop onion inbred lines (Alan et al., 2002). To be used effectively, this approach requires simple methods both for haploid plant production and chromosome doubling to obtain DH lines. The procedure is currently widely used by various breeding companies and has a major potential for basic genetic studies. Different techniques have been tried to get haploid in onion. Use of irradiation-inactivated pollen in crosses with male sterile plants was suggested as an alternative strategy for production of gynogenic onion plants (Dore, 1987). Currently, culture of immature flower buds on gynogenesis induction media slightly modified from the original protocol (Muren, 1989) is the most common method for the production of haploid onion plants. Many researchers have described improvements of the methodology, use of un-pollinated ovules, ovaries, or whole flowers, and responses of *A. cepa* lines to gynogenic induction (Alan et al., 2002; Campion and Alloni, 1990). Although recovery of haploid onion plants through gynogenesis is an established procedure, the low rate of gynogenesis in most lines, substantial plant to plant variations within lines, the low rate of spontaneous chromosome doubling, and difficulties in inducing chromosome doubling have limited the application of gynogenesis and use of DHs in onion breeding.

Along with a traditional backcross breeding program, AVRDC and CIRAD have proposed to use DH techniques to accelerate production of improved inbred lines. Here, we report acquisition of the technique and production of gynogenic plants from different generations of *A. fistulosum*-derived plants.

2 Plant material

Materials are plants generated from the interspecific cross of *A. cepa* and *A. fistulosum* (Fig.1). They come from AVRDC onion breeding program (Table 1). About 20 plants of each genotype were planted in field for flowering. Plants from the initial (F1 and F2) were not available for this experiment. But Hybrids F4 and backcross generations (BC1F1 and BC2F1) of *A. fistulosum*-derived plants (Table) were selected from the breeding program because of their resistance or tolerance to Stemphyllium leaf blight. The plant responses to Stemphyllium leaf blight had been determined by an inoculation Stemphyllium leaf blight-screening procedure. All BC2F1 plants were Stemphyllium leaf blight-resistant (R) or highly resistant (HR). *A. cepa* lines are susceptible to Stemphyllium leaf blight disease. *A. cepa* lines were fecund whereas the *A. fistulosum*-derived plants showed differences in numbers of seeds produced, with most showing lower fecundity than the *A. cepa* lines. Unlike the *A. cepa* lines,

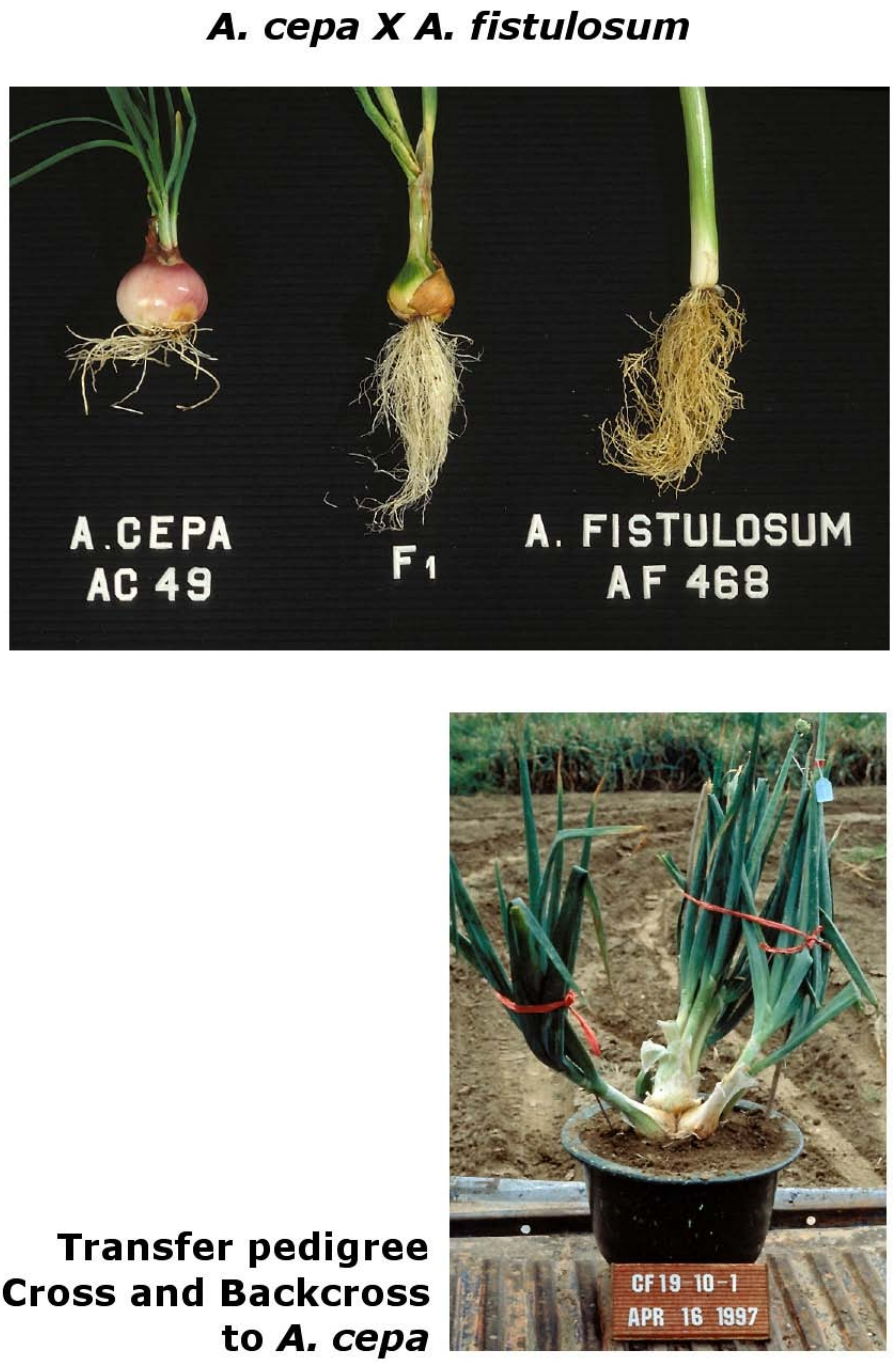


Fig.1 Interspecific cross Allium cepa X Allium fistulosum and progeny

A. fistulosum-derived plants did not produce bulbs and were not dormant. Because they did not dry down, *A. fistulosum*-derived plants were maintained in pots for months, and so, were available for flower bud collection in successive years.

3 Methods

3.1 Gynogenesis procedure

3.1.1 Preparation of flowers for culture

The whole umbel was excised, when the first flowers start to open (Fig.2). At this time about 30% flower buds have reached a three-day-before-anthesis stage (Muren, 1989). They were taken from donor plants grown in the field, between the months of December and February. The flower buds of other umbels were used on the day of collection. A few umbels were kept in the laboratory at room temperature with the bottom part of flower stalk dipped in water. In these conditions they can stay for 4 days in order to reach the stage for sampling. They were excised from the umbels. Open flowers were discarded before sterilization.

3.1.2 Aseptisation

The flower buds collected, were surface sterilized by:
- washing in ethanol 95° for 1 minute;
- sterilizing in a 2% sodium hypochlorite solution + 0.1% Tween-20 for 20 minutes;
- rinsing three time with sterilized distilled water.

Flower buds were separated into two classes: large (diameter >4 mm), and small (<4 mm).

3.1.3 In vitro culture conditions

Gynogenic induction medium: The medium used for induction of gynogenic embryos was modified B5 basal medium (Gamborg et al., 1968) supplemented with 2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/l of 6-benzylaminopurine (BA) and 75 g of sucrose (table).

Development medium (DM): This medium is a modified (Murashige and Skoog, 1962), containing, no growth regulators, and 40 g/l sucrose (Kahane et al., 1992).
All media were adjusted to pH 5.8 and were solidified with 7 g/l agar-agar (Difco-Bacto).

In vitro culture of flowers: Flower buds were placed in 90 mm × 15 mm Petri plates containing 25 ml of induction medium (20 flowers/plate). Plates were sealed with Plastic film (scello-frais) and cultured at 25 °C under cool white fluorescent and Gro-Lux lights (45–80 µEm−2s−1) with 16 h light: 08 h dark photoperiod.

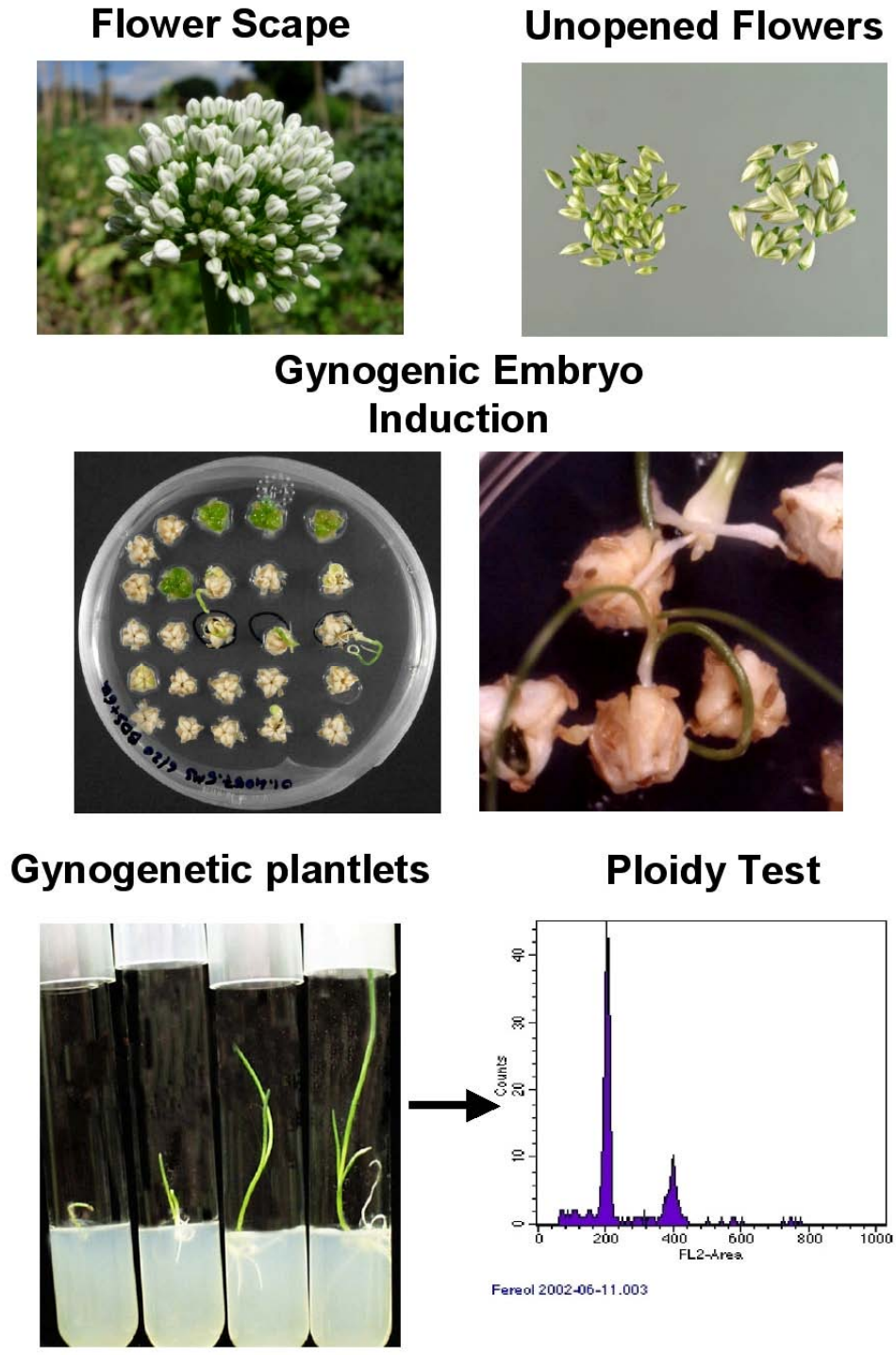


Fig.2 Sum up of the technique of induction and production of Haploid and DH Onion Plants. A-flowerscape; B-Unopened flower excised; C-Gynogenic embryo induction, gynogenic plants with seed coat; D-gynogenic plantlets emerging from ovule; E-gynogenic plantlets growing on EM medium

Table 1. Solid media used for onion gynogenesis and embryo regeneration

Nutrients (mg/l)	Induction (IM)	Development (DM)	Multiplication (MM)
Macro nutrients X 20	(B5) 50 ml	(MS) 50 ml	(MS) 50 ml
Micro nutrients X 1000	(B5) 1 ml	(MS) 1 ml	(MS) 1 ml
Fe- EDTA X 100	10 ml	10 ml	10 ml
Vitamins Morel X 500	5 ml	2.5 ml	5 ml
Growth regulators -2.4.D (1mg = 10 ml) -BAP (1mg = 10 ml) -NAA (1mg = 10 ml)	 20 ml 20 ml		 20 ml 2 ml
3.1.3.1 Divers (mg/l)	Ad SO4 40 mg	Ad SO4 40 mg KH2PO4 300 mg	L-Tyrosin 50 mg
Sucrose (g/l)	75 g	40 g	40 g
Agar-agar, Difco	7 g	7 g	7 g
PH before autoclaving	5.8	5.8	5.8

B5: Gamborg vitamins (Gamborg et al., 1968)

MS: Murashige and Skoog (Murashige and Skoog, 1962)

Vitamins Morel and Martin (1955)

AdSO4: Adenine hemisulfate C5H5N5-1/2H2SO4

Observations of the flowers culture: Observations were performed every two weeks to monitor flower development, basal callus formation and somatic shoot regeneration, and emergence of gynogenic plantlets. Basal callus development was noted. They developed the first month of culture. Structures emerging from flower ovaries cultured on induction medium are called “embryos”. Flowers responding to induction of gynogenesis were noted, and the rate of response was calculated as numbers of embryos per 100 flowers for each genotype.

In vitro sub-culture of emerging embryos : Flowers with emerging embryos were transplanted in glass tubes of development medium (DM). They grew to the 3–4 leaf stage under the conditions described above. Plantlets not growing more than one leaf were noted and eliminated. Plants in tubes were observed for overall growth performance.

3.2 Determination of ploidy of the gynogenic plants

The regenerated materials *in vitro*, and parents in the interspecific crosses *A. fistulosum* X *A. cepa* grown in soil, were analysed by flow cytometry to measure their ploidy level.

What is Flow Cytometry?

In flow cytometry (FCM-techniques) (Fig.3), a suspension of cells is passed through the path of a laser beam. Light emitted from any fluorescent material within the material transiting the beam is captured and gives rise to a signal which can be integrated over time to measure the quantity of fluorescent material within the cell suspension.

FCM is important for applications involving counting cells, or for measuring the size of cell sub-populations which have been labelled with specific fluorescent probes. FCM-DNA Ploidy works by integrating the light signal from individual passing nucleii after quantitative fluorescence labelling of DNA chromatin. In this way a conventional ploidy histogram is constructed showing population frequency of nucleii having different DNA contents (Fig.4).

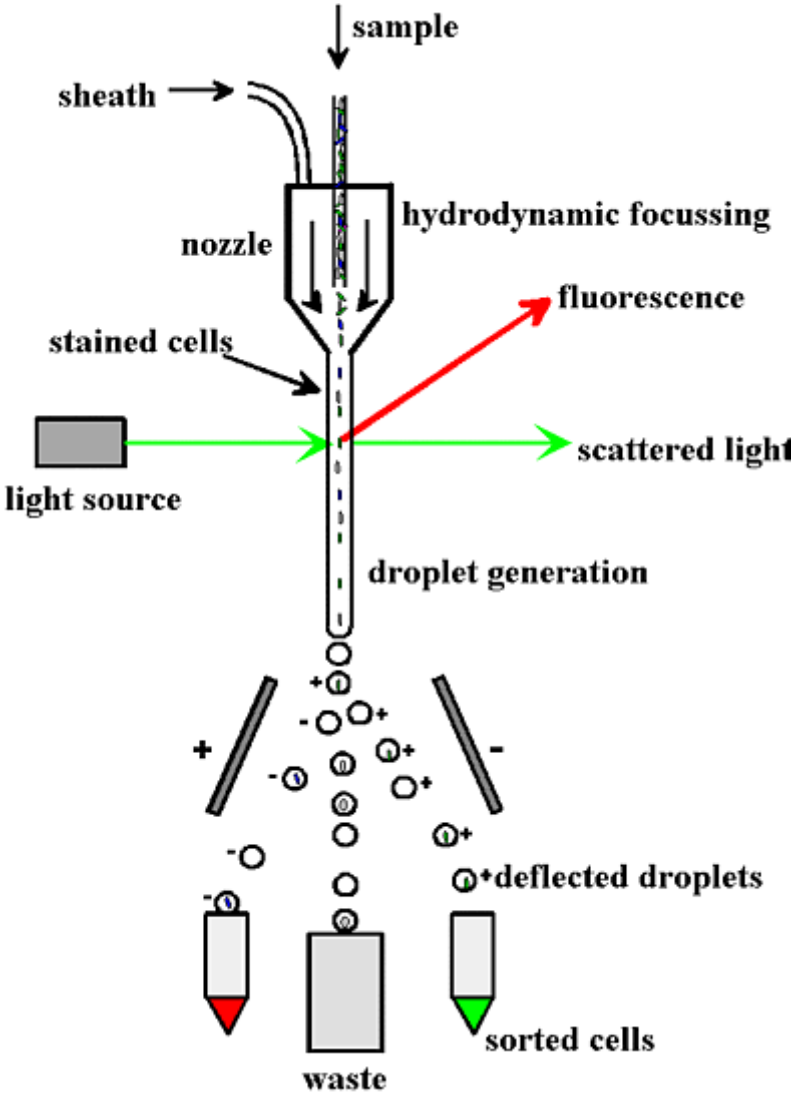
We used the protocol described by (Arumunganathan and Earle, 1991). Analyses of *A. fistulosum* and *A. cepa* plants grown in soil used the bottom parts of newly emerging leaves. Analyses of *in vitro* grown gynogenic plants (2–3 months old) used pieces of several leaves.

Approximately, 50 mg of leaf tissue was used in each sample. Leaves were cut into fine strips with a razor blade or a scalpel in 35 mm × 10 mm, polystyrene Petri dishes containing 750 µl of staining solution. Samples were filtered through a 167 µm mesh into a 50 ml Falcon tube. A second 750 µl of staining solution was added to the plate, and the filtered solution was combined with the previous one.

Samples were incubated at 37 $^{\circ}$C for 15 min and were then kept in ice until analyses were done on a FACS Calibur flow cytometer at Dr. Lei's Laboratory, Chung-Kung University, Tainan, Taiwan.

Approximately 5000 nuclei were counted for each sample. The nuclear DNA contents of diploid onion are about 32 pg/2C (Arumunganathan and Earle, 1991). Ploidy levels of gynogenic plants were assigned based on the DNA content of their nuclei in relation to the standards.

Fig. 3 A simplified illustration of Flow Cytometry is given below



3.3 Chromosome doubling

The haploid level is very stable (Jakse and Bohanec, 2000). So, it is necessary to double the chromosome number by doubling agents. Chromosome doubling of field-grown onion plants

is not possible, because the difficulty of access to the meristem. As a result, all chromosome doubling procedures in onion are based on various *in vitro* treatments of explants.

The majority of approaches attempted have used the sliced basal parts of shoots of *in vitro*-elongated or micropropagated plantlets (Fig.5), which were subjected to chromosome doubling agents (colchicine, oryzalin, trifluralin or amiprofos methyl) at various

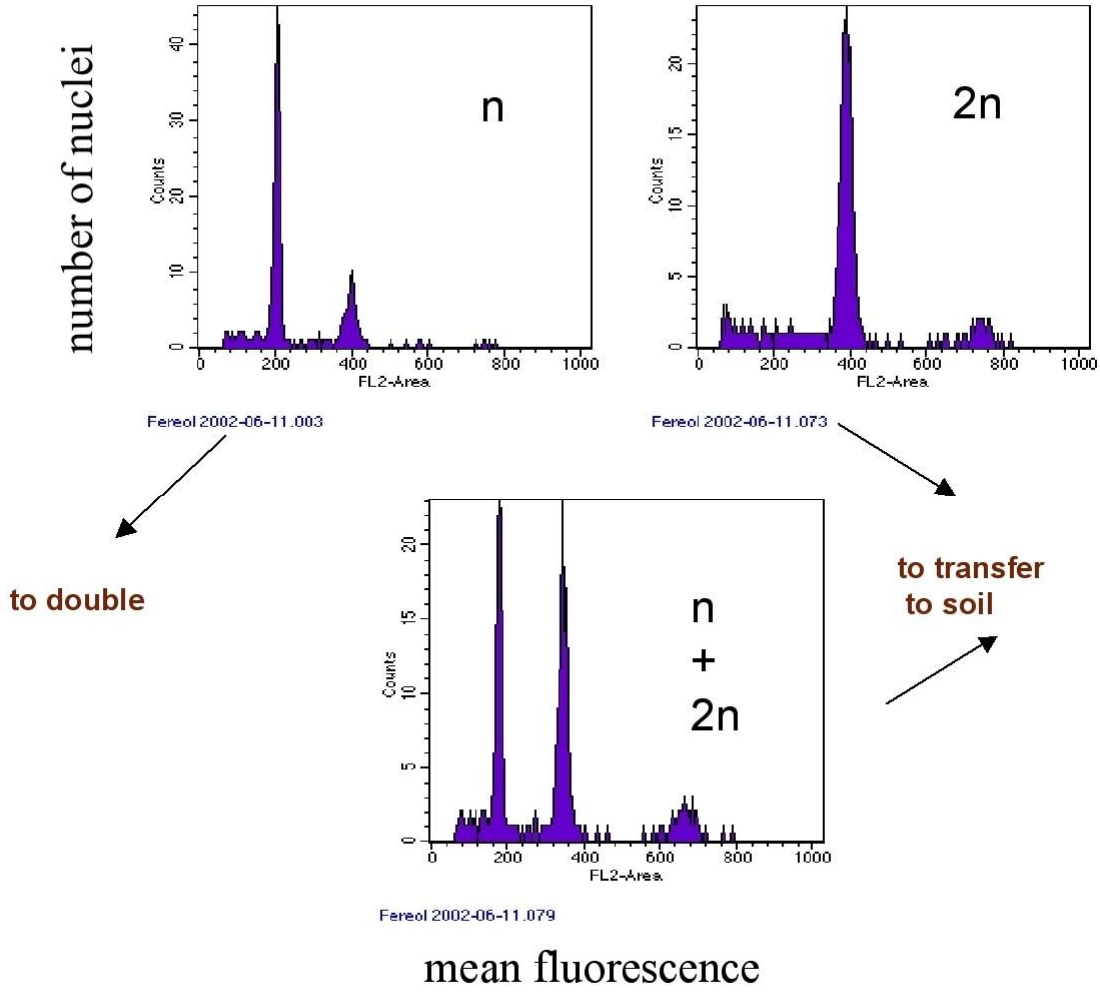


Fig.4. Flow cytometry histogram of haploid, diploid and mixoploid gynogenetic plantlets

concentrations and for various periods of exposure (Bohanec and Jakse, 1999; Geoffriau et al., 1997b; Nowak, 2000).

We tested two doubling treatments, which were colchicines and amiprofos methyl (AMP) at concentrations 25 mM/l and 50 µM/l, respectively, using application in liquid medium. Based on the results obtained in these preliminary studies APM was used in subsequent experiments. Therefore, we designed further studies using larger experimental units that included only APM as the doubling agent at concentration 50µM/l.

3.4 Transfer and observations in vivo

The gynogenic plantlets were acclimatized as follow: They were transplanted to 9 cm pots containing peat moss soil. They were covered with plastic bags, and transferred to a green house. The bags were progressively take off 1 week to help plant acclimatization and were totally removed at the end of 2 weeks. Later the plants must be transferred to field and the observed for their ability to produce bulbs, seeds and resistance to Stemphyllium leaf blight.

3.5 Maintenance in vivo of the materials

Pots of gynogenic plants, obtained from *A. fistulosum*-derived plants, will be transferred directly to field. When flowering, anthers collected from flowers will be macerated with a droplet of Alexander's stain (Alexander, 1969) and observed microscopically at 100–400 times to determine the percentage of stainable (fertile) pollen. Flowers will be self-pollinated by pollinator's house flies in covered net bags. Seeds will be collected from drying umbels and stored in paper bags.

3.6 Data analysis

Concerning production of gynogenic plantlets, the data from generations BC1F1 and BC2F1 were analyzed to determine whether the ability to produce gynogenic plantlets increased with backcrossing to *A. cepa*. Differences in the production of gynogenic embryos or plants between the BC1F1 and BC2F1 *A. fistulosum*-derived plants were tested by *T*-test. T test analysis was also used to test whether bud size class affected gynogenic plantlet formation from BC1F1, BC2F1 plants.

Concerning comparison of doubling treatments, shoot survival was visually evaluated during

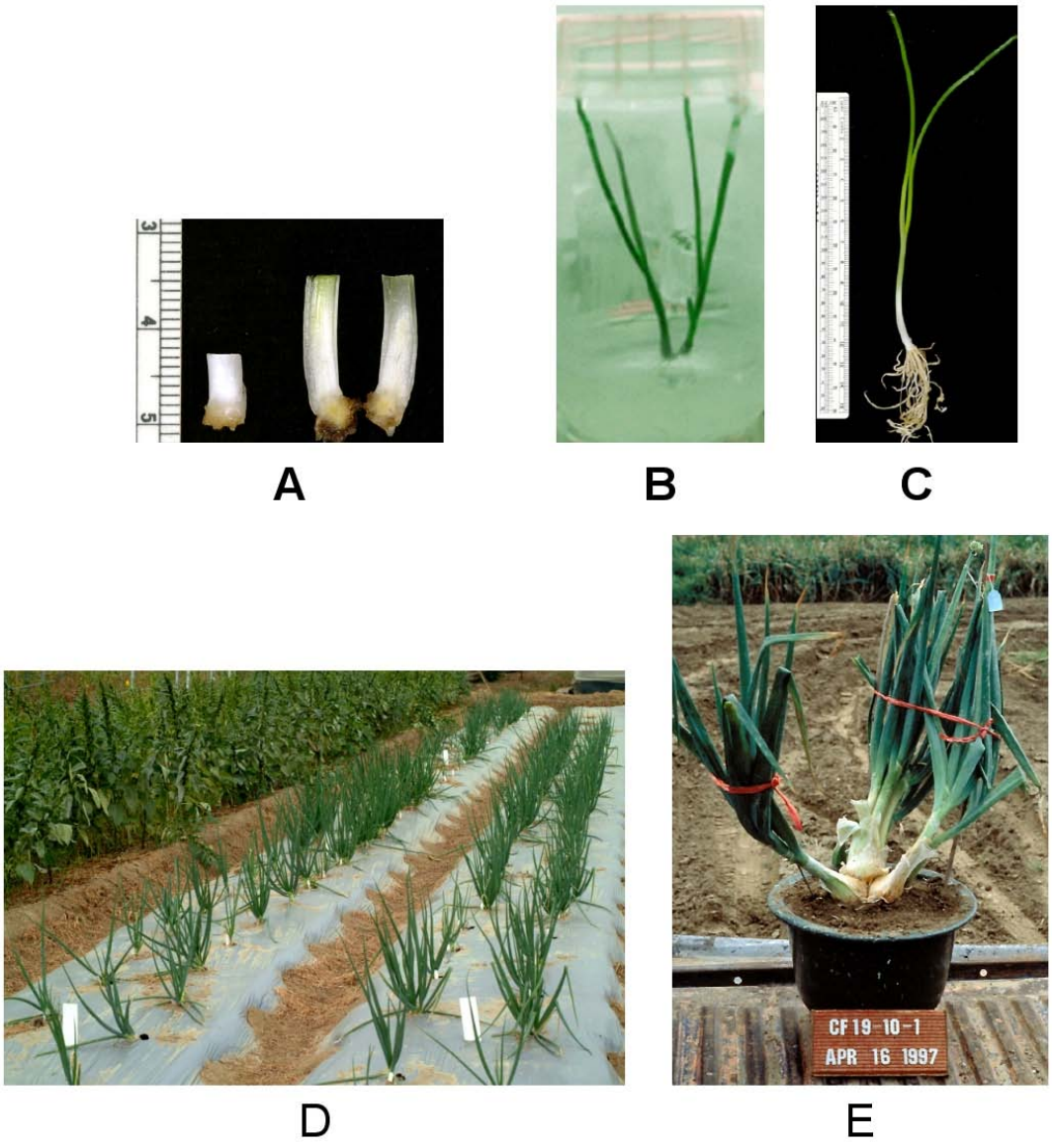


Fig.5 Straegy for double haploid of onion plants. A-anti mitotic (AMP) doubling treatment on shoot; B-Regeneration of new shoot, ploidy determination; C-Double hapoid plant; D-Transfer of double haploid to soil; E-Study of progeny

the treatment. Estimates of the percentage of shoots surviving to doubling treatment were made. Surviving plantlets are those in which the apical part of the axis, about 7-10 mm in length, remained viable and could be used for further reculturing.

4 Results

4.1 Gynogenesis induction

Gynogenic embryos formed from non-fertilized flower buds appeared gradually over a 4-month period, and development of gynogenic plantlets continued until the 16 months of culture. Hook-shaped gynogenic plants usually broke open the ovaries (Fig.2D). Their colour was either yellow or green. Many ovaries had empty black seed coats. This is consistent with the observation that all gynogenic plantlets obtained had a seed coat. Fig.2C shows a gynogenic plantlet emerging from one ovule like a germinating seedling. Gynogenic plantlets that continued to grow were transferred to tubes on EM to encourage their further development (Fig.2E).

Flower buds of *A. cepa* and *A. fistulosum*-derived plants showed similar changes in culture. Most large and small buds opened within several days of culture. Ovaries of all cultured flower buds grew to 4–8 times their original sizes after 1 month. By the end of the month, cultures initiated from different size buds looked very similar in size. Some of the flower buds were vitrified, they had a bright green and glassy appearance. Vitrification was observed in some buds from almost all donor plants. Basal callus also developed on some flower buds. The location and appearance of basal callus was quite different and distinct from that of the emerging gynogenic plantlets. Basal callus developed more frequently on small flower buds than on large ones.

4.2 Obtention of gynogenic plants

4.2.1 Responses of A. cepa line

Most of the gynogenic plantlets from this *A. cepa* line was obtained within 3–4 months although a few emerged later. Only height gynogenic plantlets (2%) were provided by this *A. cepa* line. Small *A. cepa* flower buds were less responsive to gynogenesis.

4.2.2 Responses of A. fistulosum-derived plants

F1 and F2 plants.

Flower buds from other F1 and F2 *A.fistulosum*-derived plants were not used because of problem of sterility of interspecific F1 plants.

BC1F1 and BC2F1 plants.

In 2005, we cultured 7830 flowers from 12 BC1F1 plants. We obtained 355 gynogenic plantlets from 12 responsive BC1F1 plants. Twenty five percent (25%) of plantlets were obtained from two donors plant (5380 and 5568) while the others 75% came from 9 different BC1F1 plants. The gynogenic plantlets continued to grow and were transferred to green house and then to soil to grew to maturity. Overall, response of BC1F1 plants to gynogenesis induction was low (5,28 % of all flowers cultured) but better than that of the interspecific F4 plants.

Table 2. plants used and their responses to induction of gynogenesis

Generation	**Plot**	**Fecon-**	**SLB**	**No. of flower buds**		**Gynogenic Plantlets**			
	no.	**dity**		**L**	**S**	**Number**		**%**	
						L	**S**	**L**	**S**
A. cepa									
A. cepa	**7**		**S**	**114**	**236**	**3**	**3**	**2,63**	**1,27**
Generation: F4									
F4	**5373**	**60.9**	**R**	**573**	**689**	**20**	**33**	**3,49**	**4,79**
Generation: BC1F1									
BC1F1	**5006**	**50.3**	**R**	**0**	**36**	**0**	**3**	**0**	**8,33**
BC1F1	**5366**	**48.3**	**?**	**411**	**434**	**1**	**36**	**0,24**	**8,29**
BC1F1	**5371**	**18.8**	**?**	**789**	**1084**	**1**	**52**	**0,13**	**4,8**
BC1F1	**5372**	**70.1**	**?**	**175**	**249**	**2**	**20**	**1,14**	**8,03**
BC1F1	**5376**	**55.9**	**?**	**474**	**431**	**29**	**47**	**6,12**	**10,9**
BC1F1	**5377**	**76.4**	**?**	**713**	**990**	**34**	**28**	**4,77**	**2,83**

BC1F1	**5380**	**72.2**	**?**	**232**	**333**	**24**	**45**	**10,3**	**13,5**
BC1F1	**5382**	**79.7**	**?**	**467**	**413**	**7**	**13**	**1,5**	**3,15**
BC1F1	**5563**	**64.4**	**?**	**23**	**24**	**0**	**2**	**0**	**8,33**
BC1F1	**5566**	**41.8**	**?**	**181**	**224**	**1**	**1**	**0,55**	**0,45**
BC1F1	**5567**	**74.8**	**?**	**40**	**49**	**1**	**1**	**2,5**	**2,04**
BC1F1	**5568**	**55**	**?**	**11**	**47**	**2**	**5**	**18,2**	**10,6**
Generation: BC2F1									
BC2F1	**5004**	**19.9**	**R**	**249**	**272**	**25**	**31**	**10**	**11,4**
BC2F1	**5007**	**42.6**	**R**	**20**	**16**	**0**	**8**	**0**	**50**
BC2F1	**5008**	**26.1**	**R**	**19**	**20**	**0**	**3**	**0**	**15**
BC2F1	**5026**	**54**	**R**	**344**	**515**	**20**	**63**	**5,81**	**12,2**
BC2F1	**5029**	**67.9**	**R**	**92**	**129**	**2**	**17**	**2,17**	**13,2**
BC2F1	**5031**	**34.8**	**R**	**332**	**448**	**11**	**85**	**3,31**	**19**
BC2F1	**5034**	**52.6**	**R**	**261**	**456**	**6**	**55**	**2,3**	**12,1**
BC2F1	**5072**	**60.2**	**R**	**191**	**158**	**4**	**0**	**2,09**	**0**
BC2F1	**5074**	**31.9**	**R**	**73**	**49**	**1**	**4**	**1,37**	**8,16**
BC2F1	**5075**	**37**	**R**	**194**	**111**	**22**	**11**	**11,3**	**9,91**
BC2F1	**5077**	**56.4**	**R**	**203**	**153**	**16**	**18**	**7,88**	**11,8**
BC2F1	**5092**	**98**	**HR**	**542**	**725**	**23**	**39**	**4,24**	**5,38**
BC2F1	**5095**	**58.6**	**R**	**1064**	**1704**	**30**	**93**	**2,82**	**5,46**
BC2F1	**5096**	**98.1**	**R**	**396**	**383**	**10**	**29**	**2,53**	**7,57**
BC2F1	**5097**	**96.5**	**R**	**1054**	**832**	**49**	**83**	**4,65**	**9,98**
BC2F1	**5098**	**56.4**	**R**	**810**	**659**	**31**	**75**	**3,83**	**11,4**
BC2F1	**5099**	**24.6**	**R**	**105**	**125**	**0**	**3**	**0**	**2,4**
BC2F1	**5101**	**97.5**	**R**	**309**	**604**	**17**	**76**	**5,5**	**12,6**
BC2F1	**5102**	**49.7**	**R**	**616**	**762**	**15**	**52**	**2,44**	**6,82**
BC2F1	**5103**	**32.3**	**R**	**621**	**923**	**20**	**94**	**3,22**	**10,2**

Resistance of plants to SLB was scored as follows: S: susceptible; R: resistant; HR: Highly resistant.

Table 3. Analysis of percentage of gynogenic plantlet across generations

Generation	**Flowers Cultured**	**Gynogenic Plantlets**	**Responsive Flowers (%)**	*Test t*
A. cepa	**350**	**6**	**1.7%**	
F4	**1262**	**53**	**4.2%**	
BC1F1	**7820**	**355**	**4.5%**	
BC2F1	**16550**	**1141**	**6.9%**	*0.02*

We cultured about 16539 flowers from 20 BC2F1 plants. We obtained 1141 gynogenic plantlets from 20 responsive BC2F1 plants. The plantlets obtained in 2005 grew to the plant level, and the survived transfer to soil. The plants will grow to maturity. Progress with the BC2F1 plants in 2005, encourage to culture more flowers from new and previously used BC2F1 plants in 2006.

In contrast to the results with *A. cepa* flower buds, large flower buds of *A. fistulosum*-derived BC2F1 showed significantly less response to gynogenesis induction than smaller buds. Gynogenic plants obtained from the BC2F1s were also more vigorous than those obtained from the other groups.

4.3 In vitro culture of the gynogenic plants

The gynogenic *A. fistulosum*-derived plants grew well *in vitro* on DM medium. Some contaminations in culture caused losses of many plants, in spite of attempts to rescue the contaminated plants by transfer to soil. The majority of the gynogenic plants from BC2F1 flowers showed vigorous growth *in vitro*.

4.4 Comparisons between generations

The plantlet and plant production of the BC2F1 generation was significantly greater than in the BC1F1 (Table 3). The improved response of the BC2F1 generation over the BC1F1 generation for gynogenic plant production can also be shown by direct comparison of the data from these two generations (means significantly different by two sample T-test, in the case of

small flowers).

4.5 Ploidy of the gynogenic plants

Leaves of *A. fistulosum* plants had DNA contents very similar to those of *A. cepa*. The majority of the nuclei from the *A. fistulosum*, *A. cepa*, and *A. fistulosum*-derived plants contained about 32 pg DNA. In the samples, 15–20% of the nuclei contained ca. 55 pg DNA; these nuclei may originate from cells going through mitotic division.

In this work:

- Some of the samples from gynogenic plants of *A. fistulosum*-derived plants consisted of nuclei with about 16 pg DNA, half of the nuclear DNA content of diploid onion; these were classified as haploids. These samples usually also contained a small fraction of diploid nuclei (5–25% of the total).
- Some had nuclear DNA contents identical to diploids. These plants were classified as spontaneous diploids. They represent 50% of the total.
- In other cases, samples contained similar percentages of haploid and diploid nuclei and were classified as mixoploids.
- A few gynogenic plantlets obtained from *A. fistulosum*-derived plants had nuclear DNA content of ca. 55 pg and were classified as spontaneous tetraploids.

Table 4. Flow cytometry analysis of gynogenic plants obtained from *A. fistulosum*-derived plantlets

Generation	N. Tested	n	2n	mixo-ploid.
A. cepa	3	1	1	1
F4	18	1	15	0
BC1F1	32	12	11 (34.3%)	9
BC2F1	114	27	44 (38.5%)	36

Values in paranthesis are percentages of flowers.

Mixoploid plants containing similar numbers of haploid and diploid nuclei.

4.6 Chromosome doubling

We conducted a preliminary experiment to test a method for doubling treatment. An efficient method for chromosome doubling should take in accounts both survival rate and chromosome-doubling efficiency. Concerning survival rate: The control was 100% survival, while the average survival rate for AMP (50 µM/l) or colchicine (25 mMol/l) treatments, was 27.4% and 22.4% respectively (data not shown). Concerning chromosome doubling, statistical analyses was avoid because the low number of plantlets tested may not have lead to meaningful conclusions.

Based on the results obtained in these preliminary studies on survival rate of shoots explants, and according to results by other others on efficiency of these two technique (Hansen and Andersen, 1998), we decided that, colchicine is slightly more toxic to onion tissues than APM while the doubling efficiency was similar. For this reason only APM was used in subsequent experiments following the schema Fig.5. Therefore, we designed further studies using larger experimental units that included only APM as the doubling agent at concentration 50µM/l.

5 Discussion

5.1 Production of gynogenic interspecific-derived plants

Gynogenic plants can be obtained from hybrids between *A. cepa* and *A. fistulosum.* Frequencies of gynogenic responses varied substantially among the *A. fistulosum*-derived plants. The increased plantlet production of the BC2F1 generation may indicate segregation for *A. cepa* alleles for better response to culture. It is possible that still higher levels of response would be found in more advanced generations, such as the BC2F2 or BC3F1.

The bud size of the flower is known to be an important factor in the induction of gynogenesis in onion and shallots, like (Smith et al., 1991) reported concerning leek (*Allium porrum* L.). (Campion and Alloni, 1990) re-ported that large buds (2.5–3.5 mm) were the most responsive ones to gynogenesis induction. As a general trend, culture of unopened large flower buds (corresponding to a period from several days to just prior to anthesis) is recommended (Cohat, 1994; Michalik et al., 2000). According to (Bohanec and Jakse, 1999; Geoffriau et al., 1997a), flower buds just prior to anthesis contain fully mature embryo sacs, which may be a contributing factor to the higher frequencies of gynogenesis in large flower buds. But here, like previous workers, we found no significant difference between large and small flower buds. So, we can included all size flower buds in the culture of *A. fistulosum*-derived

generations.

5.2 Ploidy level

The majority of gynogenic plants obtained from *A. fistulosum*-derived plants here were diploids. Occurrence of various rates of spontaneous mixoploids, diploids, and tetraploids among gynogenic onion plants has been reported by others (Bohanec and Jakse, 1999; Geoffriau et al., 1997a). However, the high rate of spontaneous diploids among the gynogenic plants from *A. fistulosum*-derived plants is similar to result obtained by Alan et al. (2003) for A. roylei-derived plants. We assume that gynogenic plants with ploidy levels other than haploid resulted from spontaneous chromosome doubling in early stages of egg cell division and/or embryo development, but the possibility of their development from unreduced egg cells should not be disregarded. Therefore, study of their self-pollinated progenies or molecular analyses would be desirable to confirm their homozygosity. If truly of haploid origin, these diploids are advantageous, since they eliminate the need for chromosome doubling procedures.

A large proportion of plantlets from all treatments expressed mixoploidy, a phenomenon that was also noted in previous reports (Geoffriau et al., 1997b). It is not yet clear whether mixoploid regenerants develop into partially fertile diploid plants or remain haploid, as suggested by (Geoffriau et al., 1997b). In the case of partial diploidy, at least some of them might produce seeds.

5.3 Chromosome doubling

The choice of APM as the optimal chromosome doubling substance is in agreement to similar findings in other plants. (Hansen and Andersen, 1998) reported that compared to oryzalin and trifluralin, APM showed a relatively low toxicity on gynogenic embryo formation in sugar beet. APM was also shown to be less toxic but similarly efficient when these three substances were compared in Brassica napus microspore cultures (Hansen and Andersen, 1998) and less inhibitory to maize haploid callus growth than oryzalin (Wan et al., 1991). Due to its low toxicity and high-doubling efficiency APM could be the optimal choice among the most frequently tested chromosome-doubling substances.

5.4 Use in onion breeding

In several crops, double-hapoid plants have been of great utility. First for the rapid generation of inbred lines for hybrid production and then for the creation of recombinant inbred lines (RILs), so useful for developing molecular markers, mapping genes of interest, and detecting QTL affecting quantitative traits. When working with a trait that is difficult to screen on a single plant basis, generation and evaluation of fixed inbred materials is also of considerable benefit. Doubled haploids derived from interspecific materials, could have additional advantages as well. Using an efficient gynogenic system in moderate to advanced interspecific generations would generate RILs fixed for novel traits but containing relatively little wild genomic content, significantly accelerating the breeding process.

Breeding to transfer the traits of disease resistance of *A. fistulosum* to cultivated onion has been impeded by sexual barriers between the species that reduce fecundity in cross and backcross generations (Kik, 2002). Nevertheless gynogenic plants can be obtained from breeding material generated from interspecific crosses of onion and *A. fistulosum,* allowing development of fecund inbred lines from plants at BC1F1, and BC2F1 stages. There was no apparent association between the fecundity of the *A. fistulosum*-derived donor plants and the ability of the buds to produce gynogenic plantlets (Table 1). DH *A. fistulosum*-derived plants could be of material assistance in circumventing the sexual barriers in the transfer of traits such as disease resistance from *A. fistulosum* to onion.

The percentage of gynogenic plantlets increase as generations of *A. fistulosum*-derived plants were advanced. This indicates the existence of genetic effects in gynogenesis induction. As the share of *A. fistulosum* DNA in the resulting plants decreases, their gynogenic response is likely to improve as well.

The next step in this project will be attempts to recover self-pollinated seed from the spontaneous and induced diploids. Seeds of the gynogenic *A. fistulosum*-derived BC2F1 plant will be grown in the later season, and the resulting seedlings will be tested for stemphyllium resistance and advanced in the breeding program as appropriate.

Unlike those obtained from *A. cepa* plants, the majority of the gynogenic plants we obtained from *A. fistulosum*-derived plants were spontaneous diploids, not requiring chemical doubling. Recovery of self-pollinated seed from such plant would suggest that some of the plants may be fecund. If homozygous and fecund, these plants may speed development of new onion cultivars with useful agronomical characters such as resistance to stemphyllium. DH plants currently being obtained from more advanced backcross generations may be even more valuable to the breeding program.

The procedures developed for common onion are also applicable to shallot (*A. cepa* L. var. aggregatum) (Cohat, 1994; Sulistyaningsih et al., 2002).

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7 Annexe 1. Stock Solutions of media used for haploid production of onion

Macro nutrients	**(B5)**		**(MS)**			
Concentration	Final Sol mg	X 20	Final Sol	X 20		
Quantity stock solution per liter of final solution		50 ml		50 ml		
Quantity of stock Solution to do		**1 liter**		**1 liter**		
-KNO3	2500mg	50 g	2500	50g		
-(NH4)2SO4	134	2.68g				
-NH4H2PO4			300	6g		
-KH2PO4, 2H2O			170	3.4g		
-NaH2PO4, 2 H2O	150	3g				
-CaCl2, 2H2O	150	3g	200	4g		
-MgSO4, 7H2O	250	5g	400	8g		
Micro nutrients	**(B5)**		**(MS)**			
Concentration	Final	X 500	Final	X 1000		
Quantity mother Sol for 1 l of final Sol		1 ml		1 ml		
Quantity of mother Sol to do		**1 liter**		**1 liter**		
- H3BO3	3mg	3000mg	6,2mg	6200mg		
- KI	0.75	750	0,83	830		
- MnSo4, 4H2O	10	10 000	16,7	16700		
- ZnSo4, 7H20	2	2000	8,6	8600		
- CoCl2, 6H2O	0,025	25	0,025	25		
- CuSo4, 5H2O	0,025	25	0,025	25		
- Na2MoO4, 2H2O	0, 25	250	0, 25	250		
Vitamins	**(Morel)**					
Concentration	Final	X 200				
Quantity mother Sol for1 liter of final Sol		5 ml				
Quantity mother Sol to do		**500 ml**				
- Nic ac	1	100 mg				
- pyridoxin	1	100				
- Thiamine HCl	1	100				
- Calcium panthotenate	1	100				
- biotin	0.01	1				
- myo inositol	100	10 000				
Iron EDTA						

Concentration Quantity mother Sol for 1 l of final Sol	Final Sol	X 100 10 ml	
Quantity mother Sol to - FeSO4, 7H2O - Na2 EDTA	27.8 mg 37.3	**1000 ml** 2.785 g in 500 ml H2O, 3.725 g in 500 ml H20,	Pour Na2 EDTA heat, in FeSO4 heat, progessively to avoid
Growth regulator			
- 2.4.D	1mg = 10ml	50mg+ 2,5ml alcohol 90° + 500ml H2O	
-NAA	1mg = 10 ml	50mg+ 1 pastille KOH+ few H2O: dissolve, then complete500ml H2O	
- BAP	1mg = 10 ml	50mg+ 1 pastille KOH+ 500ml H2O	

8 Annexe 2. Flow cytometry protocol

Flow cytometry

Protocol for the extraction and coloration of nuclei,

Adaptation to *Allium sp* leaves

Extraction of nuclei

About 250 mg (~ 4 cm^2 of leaves) of sample are finely chopped by a razor blade, in a Petri dish, in 0.7 ml of buffer PBS (**A**).
The suspension, obtained like this, is filtrated through a 48 µm pore sieve on flow tube.

Colouring

The filtrate is coloured by a solution of Propidium Iodide (**B**), 200 µl by tube.
Stir by Vortex Shaker, and incubate at room temperature for 5 mn.
Stir again before taking each measure.

Preparation of solutions

A: Solution of PBS

To this, add:

- Dithiothréitol (DDT), Sigma réf. D5545 1mg per ml of buffer
- Triton X100 6 ml in 100 ml of buffer

Dissolve DDT before adding Triton X100
Stock at +4°C for stock and during reading of results

B: Propidium Iodide

Prepare a watery solution 1 mg/ ml of water. (Can be stock 2 months at 4°C).
For colouring, dilute this solution to 1/5 (3ml PI at 1mg/ml + 12 ml of buffer A). Maintain at +4°C (for stock and during utilisation).

Caution of use: CHEMICALS DANGEROUS

Don't put in contact with skin
Labelled the bottles well
Throw away wastes in a container used for this purpose.

POTENTIALITIES OF GARLIC SHOOT TIP AND SOMATIC EMBRYOGENESIS IN THE CONTEXT OF THERAPY AND GERMPLASM PRESERVATION

1 BACKGROUND INFORMATION

Dissemination of improved or selected varieties of garlic (*Allium sativum* L.), to farmers, is hampered by the presence of two viruses belonging to the potyvirus group, Onion Yellow Dwarf (OYDV) and Leek Yellow Stripe (LYSV) (Lot et al., 1994; Van Dijk, 1991; Van Dijk, 1993; Walkey, 1990), and in obtaining healthy plants. Moreover, the need for successive multiplication phases in field in order to provide sufficient material hampers the rapid dissemination of selected elite material. The **establishment of a virus free plant production system** is a prerequisite for seed production and dissemination. The traditional scheme usually involves virus-free stock material, micro propagation technique, seed production, certification and distribution of high sanitary quality materials. Some viruses can be effectively eliminated from infected plants owing to their mode of replication and their mechanism of movement within the plant.

Three methods are currently used: thermotherapy, meristem tip culture and chemotherapy. According to the literature, meristem culture is considered to be the reference tool for virus disease eradication (Faccioli and Marani, 1998). This technique takes advantage of the fact that **many viruses fail to invade the meristem region**. Transfer of the meristem dome, together with one or two leaf primordia, to a culture medium and development into a plantlet may lead to the elimination of a virus. There are reports of the elimination of sugarcane yellow leaf virus (SCYLV) using meristem culture (Chatenet et al., 2001; Fitch et al., 2001; Parmessur and Dookun, 2000). Concerning garlic, eliminating viruses and producing virus-free material have been developed for the past 30 years, using meristem-tip culture (Chovelon et al., 1990; Walkey et al., 1987). It was found that a gradient of increasing virus concentration from the dome to the successive primordia exists (Walkey and Webb, 1968). This means that the possibility of obtaining virus-free plants is inversely related to the size of the meristem excised. However, the virus-free state of plants regenerated after meristem culture alone is not efficient at 100%, particularly when meristems were excised from *in vivo* plants comparatively to *in vitro* plants.

To improve pathogen elimination, thermo and chemotherapy coupled with meristem culture can be used when meristem culture alone fails. Virus elimination was greatly accelerated at 40°C, but with a subsequent deterioration of the cultured tissue. Chemotherapy has also been applied to meristem cultures, but this has a negative effect on meristem growth.

Therefore, other technique with less negative effect might be attempted. Somatic embryogenesis, was shown to be highly effective in the elimination of certain viruses from *Vitis vinifera* (Goussard et al., 1991), *Saccharum officinarum* (Parmessur et al., 2002). The availability of a reliable technique for the production of somatic embryos from garlic (Fereol et al., 2005a; Fereol et al., 2005b) prompted us to investigate whether the use of this technique could result in the elimination of viruses OYDV and LYSV from Garlic.

In the present study we report the establishment and regeneration of embryogenic cell suspension cultures of an infected garlic cultivar. We assess and compare infection at three steps of this process: embryogenic callus, suspension cultures, and on plantlets regenerated from these different tissues type.

2 PLANT MATERIAL

2.1 Cultivar

The cultivar “29 VFG” (AVRDC, Allium Unit), one part grown in open field and, one part grown in net house was used in this experiment. It belongs to the tropical group of garlic, short-day type, expressing short dormancy and bad preservation. It forms medium-size bulb with white coloured cloves. This variety was issued from clonal selection and meristem-tip culture. Bulbs, harvested from plants 4-5 months old, were stored 3 weeks at 5°C, in order to break the dormancy before experiments. Cloves from these bulbs were germinated *in vitro* according procedure developed by (Kahane et al., 1992). Plantlets issued of this *in vitro* culture were tested by ELISA test for the presence of OYDV and LYSV. On one side, *in vitro* cultivated materials coming from net house and tested virus-free, and on other side *in vitro* cultivated materials coming from open field and tested infected by both OYDV and LYSV were used for somatic embryogenesis.

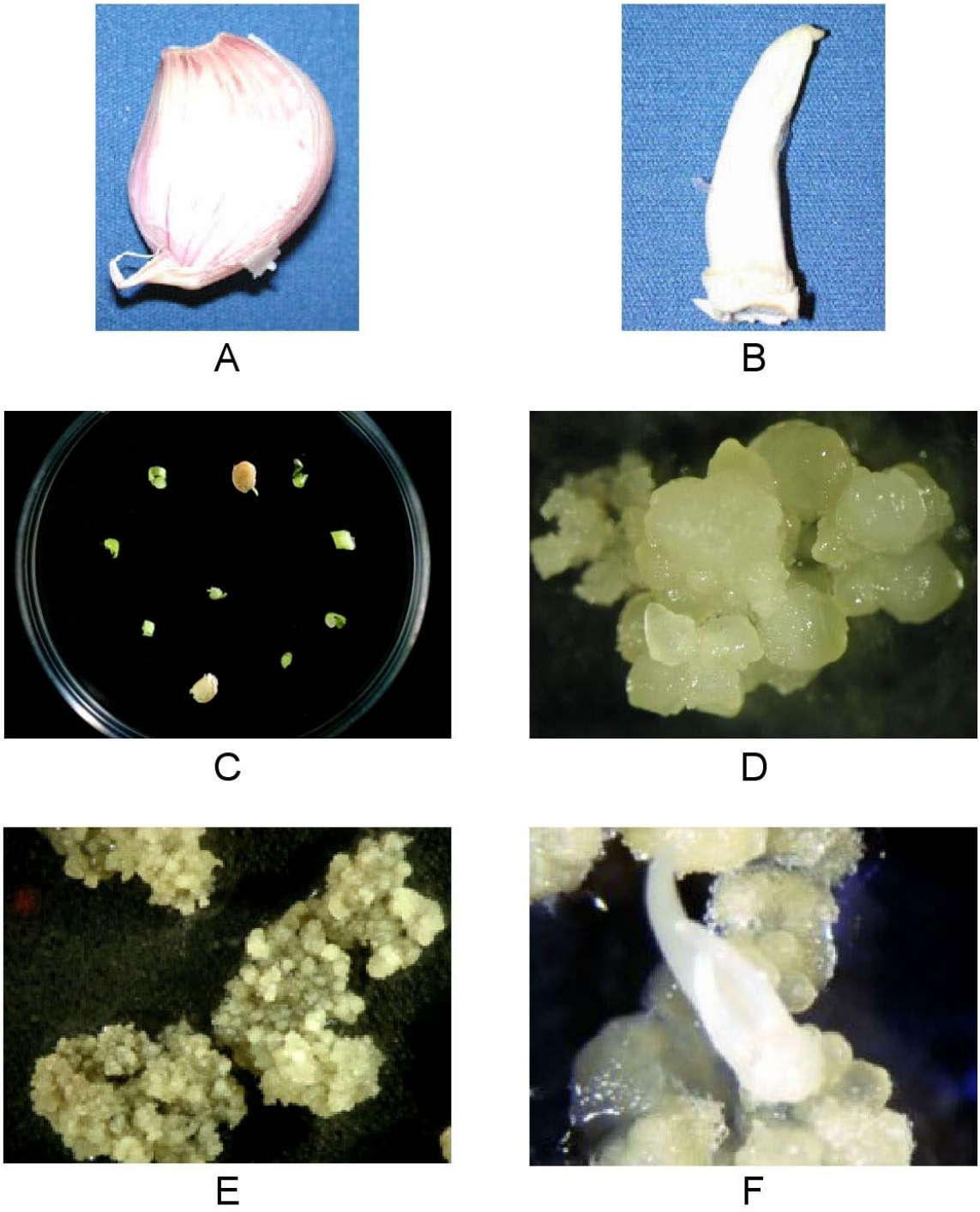


Fig. 1 Callus culture; A-clove; B-shoot from clove; C-shoot roll explant; D-primary callus; E-induction of embryogenic and friable callus; F-regenerated embryo

2.2 Explants

Roll shoot sections including young leaf part and basal plate was used as explants for callus induction (Fig.1A-C).

Cloves were surface sterilised according the following procedure: After removing the outer protective leaf sheaths, they were dipped in 70% ethanol for 5 minutes, followed by 20 minutes in a sodium hypochlorite solution (2% of active chlorine). They were then rinsed three times in sterilised water for 5, 10 and 15 minutes. They were dried on a sterile paper towel, the storage leaf was removed under aseptic conditions. Then the shoot containing the basal plate and basal young leaf part was cut transversally to produce roll shoot explants 2 mm thick.

3 METHODS

3.1 Callus production

The explants were plated on 90 mm diameter Petri dishes containing 25 ml of semi-solid medium. They were incubated 1 month on a callus pre-induction medium (CIM1, Tab.1), which is a modified N6 medium (Chu et al., 1975) containing 0.2 mg l^{-1} NAA, 0.1 mg l^{-1} IAA, 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP, and solidified with 0.3% agar (Phytagel®). The explants were then transferred to a second induction medium (CIM2, Tab.1) for callus production. This medium differed from the previous one by concentrations of 2,4-D (0.5 mg l^{-1}, Tab. 2). The callus produced were transferred on a maintenance and Embryogenic medium (CMM, Tab.1) based on a N6 medium containing cefotaxime (400 mg l^{-1}), and maintained on it before embryo induction or initiation of suspension cultures.

3.2 Regeneration from calluses

Callus issued from CMM were transferred on an embryo induction medium (EIM, Tab.1) containing 2,4-D/kinetin (0.05/2.5 mg l^{-1} respectively). The cultures were incubated at 24-26°C in darkness for two months. These callus differentiated globular somatic embryos after 2 months, and they were transferred to an embryo development and germination medium (EDM, Table 1) which is a SH modified medium containing BAP (0.7 mg l^{-1}) and ANA (0.2 mg l^{-1}) for two months (Fig.1F).

3.3 Initiation of the suspension cultures

The embryogenic calluses were sub-cultured repeatedly every 30 days on callus maintenance medium (CMM, Table 1). From 5 months, they formed a mixture of compact, semi-friable and friable calluses (Fig.2A). Such calluses were used to initiate cell suspension cultures. Callus (0.25g) was cultured in each well of 6x10 ml multi-well dishes (Fig.2B) containing 5 ml/well of liquid suspension medium (SM, Table 1) based on N6-modified salts (Chu et al., 1975). The cultures were incubated at 24-26°C in the dark in continuing agitation (100-110 rpm). After 14 days, 3 ml of the medium were removed and replaced by 3 ml of fresh medium. Sub-culture was performed every 28 days by transferring half of the callus culture and all the fine suspension in a new well. After 3 to 4 months since the beginning of the callus culture in liquid medium, a suspension was initiated.

3.4 Establishment and maintenance of the cell suspension culture

The cell suspension cultures initiated above were sub-cultured in multi-well dishes, by transferring half of sediment cell volume (SCV) in 2 ml of the previous SM medium, in a well to which 3 ml of fresh SM medium were added to give an initial volume of 5 ml.

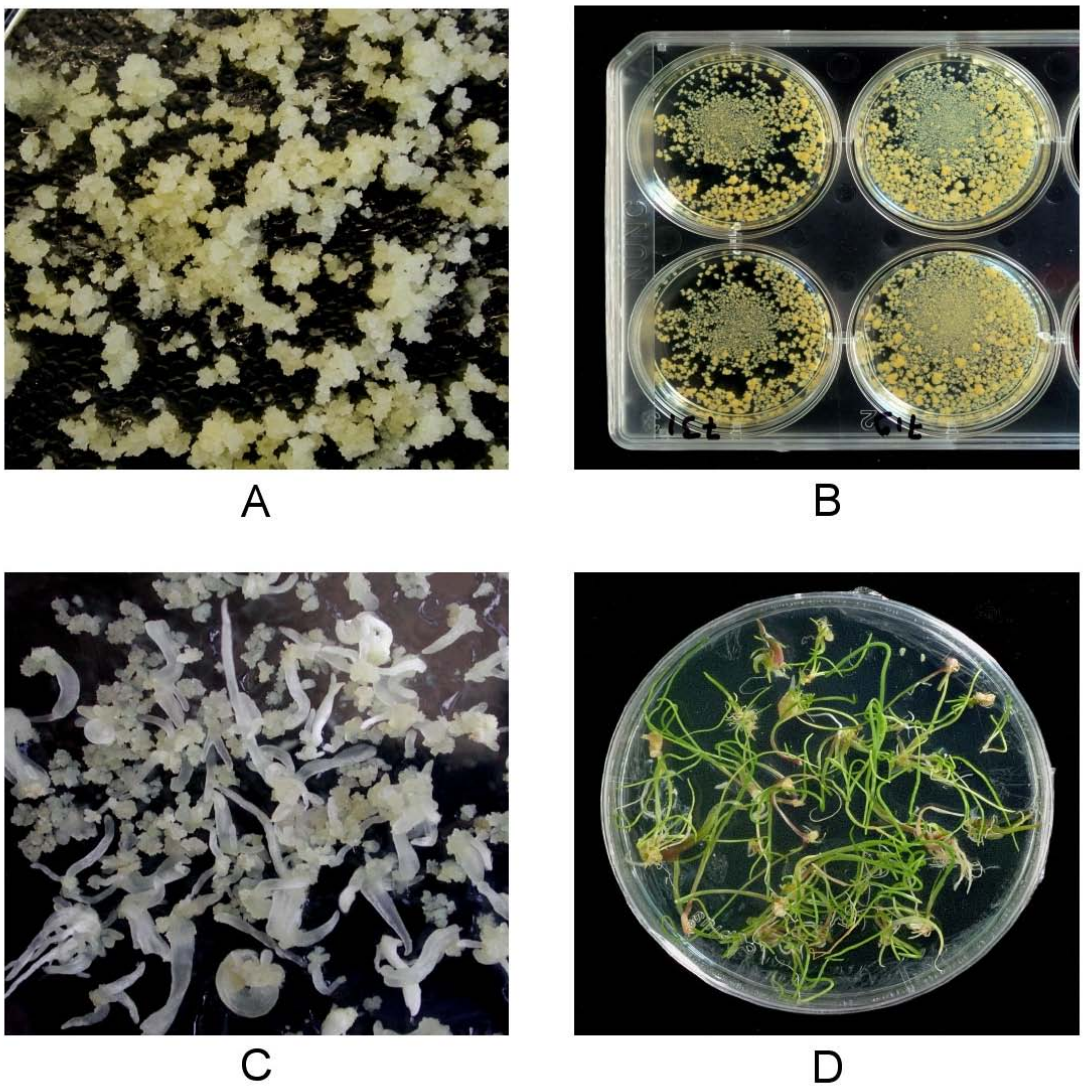


Fig.2 Establishment and maintenance of suspension culture A-Friable and embryogenic callus; B-initiation of suspensionc ulture in multi-well dish; C-regeneration of embryos after plating on semi-solid medium; D-conversion of embryos into plantlets.

Table 1 - Solid and liquid media used for garlic embryogenic suspension cultures and embryo regeneration.

Nutrients (mg/l)	CIM1	CIM2	CMM	SM	EIM	EDM	PGM
Macro nutrients	(N6) 50 ml	(N6) 50 ml	(N6) 50 ml	(N6) 50 ml	(SHm) 50 ml	(SHm) 50 ml	(SHm) 50 ml
Micro nutrients	(MS) 1 ml	(MS) 1 ml	(MS) 1 ml	(MS) 1 ml	(MS) 1 ml	(MS) 1 ml	(MS) 1 ml
Iron EDTA	(MS/2) 5 ml	(MS/2) 5 ml	(MS/2) 5 ml	(MS/2) 5 ml	(MS) 10 ml	(MS) 10 ml	(MS) 10 ml
Vitamins + amino acid	(L2) 5 ml	(L2) 5 ml	(L9) 20 ml	(L9) 20 ml	(SHm) 5 ml	(SHm) 5 ml	(SHm) 5 ml
Other amino acid (mg) - Prolin - Glutamin	230	230	230	230	150	150	
Organic substances Myo-inositol	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	
Growth regulators -2.4.D -AIA -ANA -kinetin -BAP - cefotaxime	 10 ml 1 ml 2 ml 1 ml	 5 ml 1 ml 2 ml 1 ml	 5 ml 1 ml 2 ml 400 mg	 3 ml 1 ml 5 ml 400 mg	 0.5 ml 1 ml 5 ml 25 ml	 0.5 ml 1 ml 2 ml 7 ml	
Sucrose (g/l) Manitol (g/l)	40 20	40 20	40 20	40 20	20 40	20	20
Agar, Phytagel (g/l)	3	3	3g	3g	3g	3g	3g
pH before autoclaving	5.8	5.8	5.8	5.8	5.8	5.8	5.8

CIM: callus induction medium; CMM: callus maintenance medium; EIM: embryo induction medium; SM : suspension medium; EDM: emlbryo development medium; PGM: Plant growth medium; SHm: SH modified.
N6 basal medium (Chu et al., 1975).
(MS) Murashige and Skoog medium (Murashige and Skoog, 1962).

Several parameters must be taken into account for maintaining the suspension cultures and guarantee the embryo production:

3.4.1 Initial culture densities

The quantities of SCV cultured in 5 ml of SM medium. Half of the sediment cell volume (SCV) after 28 days of culture measure about 0.24 ml.

3.4.2 Periodicity of medium renewal

The periodicity of medium renewal was the interval of 14 days between two successive medium renewals.

3.4.3 Age of the suspension cultures

From 1 to 15 months after initiation, the medium was renewed every 14 days and sub-culture was performed every 28 days.

3.5 Regeneration of the suspension culture

The regeneration potential of cell suspension cultures was studied by plating on semi-solid embryo induction medium (EIM, Table 1), as for regeneration from calluses. Two months later, these cultures (Fig.2C) were collected from EIM and cultured on an embryo development and germination medium (EDM, Tab.1) to develop whole plants (Fig.2D). This SH medium was supplemented with BAP (0.7 mg l^{-1}). Such regenerated plants were transferred on a plant growth medium (PGM, Tab.1). The *in vitro* plants were further developed *in vitro* according to the protocol reported by (Kahane et al., 1992).

3.6 Adaptation to in Vivo Conditions

Acclimatization was performed at rooted stages. After rinsing with sterile water, the *in vitro* plants were established in compost soil and gradually hardened in greenhouse. The adaptation

of *in vitro* propagated and rooted plants to non sterile conditions was done in a steel-glass greenhouse under air conditioner.

3.7 ELISA Test

The sanitary state of the materials was checked through DAS-ELISA with the DSMZ (Deutsche Sammlung von Mikrooganismen und Zellkulturen, Gmbh) kit for the detection of Onion Yellow Dwarf Virus (OYDV) and Leek Yellow Stripped Virus (LYSV), according to the manufacturer's instructions (annexe 2). The different materials, pieces of leaves from plantlets, or callus, and or sediment culture of suspension cultures, (0.4—0.5 g for each) were homogenized in 10 volumes of extraction buffer containing 0.5 ml Tween-20, 20 g PVP10 (polyvinylpyrolidone) and 2 g bovine serum albumin at pH 7.2. The samples were used directly. Tissue samples from healthy and infected mother plants provided by this company were used as negative and positive controls. Absorbance at 405 nm (A405) was read in an ELISA microplate reader. The A405 values of healthy controls ranged from 0.00 to 0.05 and values above 0.30 were considered as positive.

3.8 Virological Investigations

A flow chart (Fig.3) summarizes this experiment. A total of 80 *in vitro* germinated shoots (Fig.3-A-D), were tested for the detection of Onion Yellow Dwarf Virus (OYDV) and Leek Yellow Stripped Virus (LYSV). They came from 40 bulbs harvested in open field and 40 from plants maintaining in net house. Each *in vitro* germinated shoot correspond to one distinct bulb and clove per bulb.

To determine the stage from the process for elimination of the virus, the same tests were perfomed on callus (Fig.3-E), suspension cultures (Fig.3-G) and plantlets in green house (Fig.3-F and H) issue of these calluses and suspension cultures. We tested 6 samples of calluses, 6 of suspension culture, 36 plantlets issue of callus-derived somatic embryos, and 36 plantlets issue of suspension cultures-derived somatic embryos.

The putative virus-free plantlets will be tested a second time after a net house transfer phase.

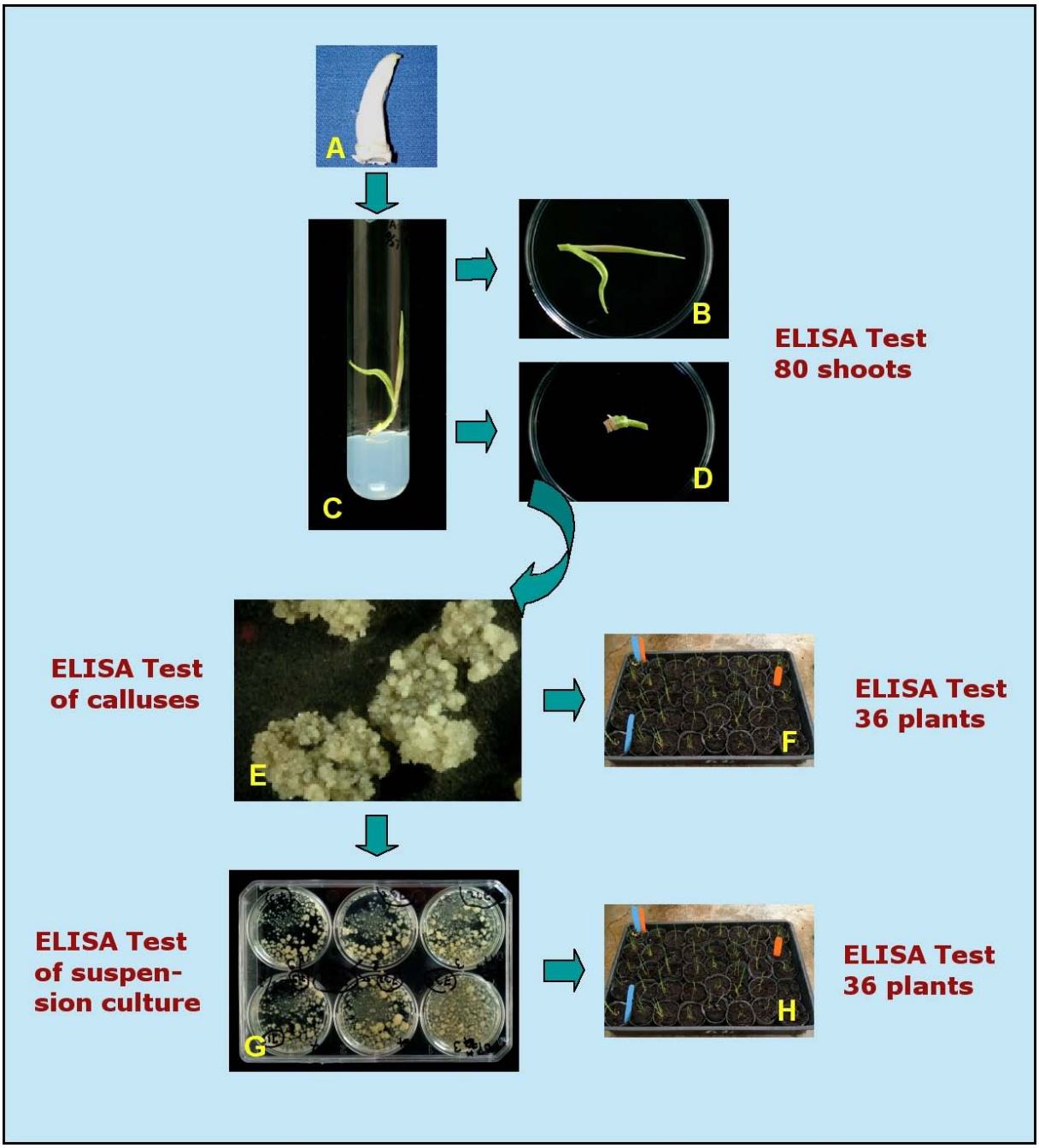


Fig. 3 Flow chart of the experiment, A-shoot froù clove; B-*in vitro* germinated shoot; C-leave from « B » for testing virus status; D-shoot from « B » for initiation callus; E-friable embryogenic callus for initiaion suspension culture and regeneration embryo-derived plantlets (F); G-suspension culture for regeneration embryo-derived plantlets (H)

The eradication rate (ER) on plantlets observed *in fine* for each phase of the process will be calculated as follows:

- First ELISA test performed on *green house* plants:

ERi= (negative green house plants/tested green house plants) X 100

- Second ELISA test performed on net house plants:

ERf= (negative green house plants/tested green house plants) X (negative net house plants/tested net house plants) X 100

3.9 Recording

Observations on the following variables were performed:

- number of explants with callus on CIM2,
- number of callus containing embryogenic tissue after 6 months on CMM,
- number of friable and embryogenic calluses
- number of somatic embryo derived plantlets.
- measure of the SCV rate (final SCV / initial SCV), determining the growth of suspension cultures during maintaining phase
- number of regenerated somatic embryos derived plantlets per aliquot of 0.1ml SCV from each replicate.
- presence or no of viruses at each stage of the process (callus, suspension cultures, plantlets regenerated from callus and from suspension cultures).

3.10 Statistical analysis

All data were analysed using SAS statistical package (SAS, 1982) version 8.2 for windows. The experimental design consisted of 1 cultivar, 2 original virus status, originated virus free (V-) and originated virus infected (V+), 6 replicates of 10 explants by original virus status.

The t-test procedure was performed for the different variables. The means were compared using the two-sample t-test procedure.

4 RESULT

4.1 Callus production

After one month on CIM1, the explants increased in size but no callus formation was observed. These explants sub-cultured on CIM2 for one month more, started producing callus translucent, nodular type, and some time yellowish, also nodular (Fig.1D-E). These calluses were generally located at leaf edges near a vein. Clumps of callus of both aspects were transferred on CMM medium. According to t-test, no significant difference was found between the virus statuses at 5% level (Table 3).

Table 3. Comparative callus induction from two original virus status

Type of callus induction %	**original virus status**		*T test*
	Vrirus (-)	**Virus (+)**	
Total number of explant	**60**	**60**	
Primary callus	**49**	**52**	*0.45 NS*
Callus with embryo-genic tissue	**29**	**27**	*0.71 NS*
Friable and embryogenic callus	**14**	**11**	*0.55 NS*

NS: no significant difference between the original virus status at 5% level by T test

4.2 Embryo induction from calluses

After two months on EIM, the embryogenic callus formed globular somatic embryos (Fig.1F). The OYDV and LYSV infection of the donor plants had no significant effect on the production of callus and callus showing embryos (Table 4).

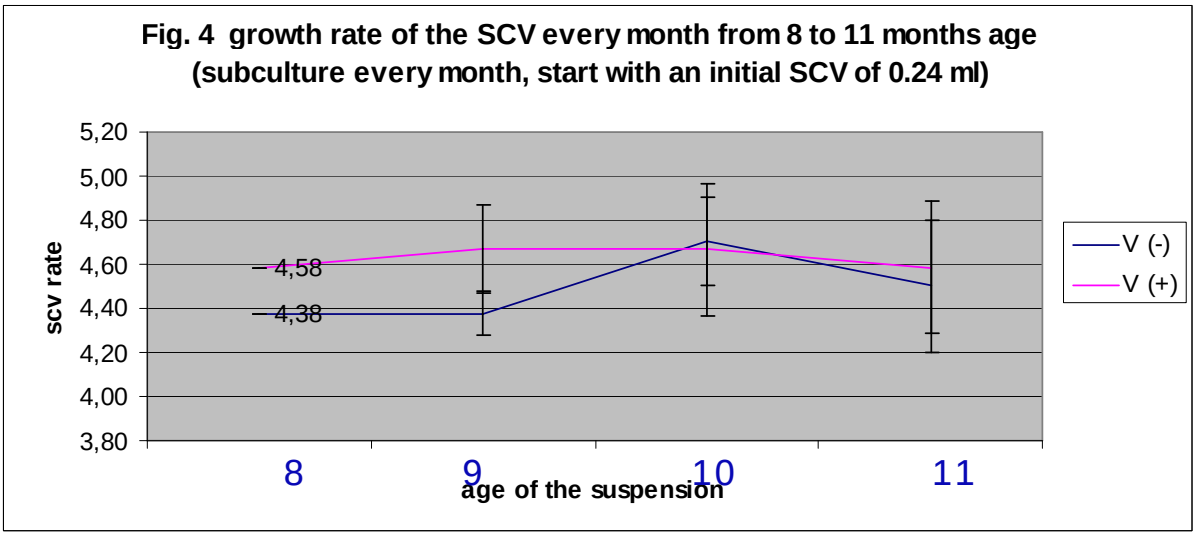
Table 4. Regeneration of callus function of virological status

Type of callus induction %	original virus status		*T test*
	Vrirus (-)	Virus (+)	
Total number of petri dishes	6	6	
Mean Nb. of plantlets regenerated per replicate	31.5	29.3	*0.49 NS*

NS: no significant difference between the virus status at 5% level

4.3 Initiation of the suspension cultures

The growth of the cell suspension culture was on average quasi similar up to 8 month age. The OYDV and LYSV infection of the donor plants had no significant effect on the growth rate of the suspension cultures.



4.4 Regeneration of the suspension culture

Aliquots of cell suspension, plated on semi-solid embryo production medium (EIM, Table 1), could produce numerous somatic embryos in 8 weeks (Fig.2C). They looked like large structures (2-5 mm) with a smooth surface due to the presence of an epidermis. The best combinations of growth regulators were 2,4-D/kinetin (0.05/2.5 mg l^{-1}).

4.5 Conversion of somatic embryos into plants

The germination process occurred within 4 weeks. The embryos subsequently developed the shoot and the root apex, and formed a complete plantlet (Fig.2D). The addition of BAP (0.7 mg l^{-1}) increased it up to 30%. With 0 to 0.5 mg l^{-1} BAP, the plants presented a normal development, but higher concentrations (2 mg l^{-1}) promoted abnormalities like curled and dark green leaves or multiple shooting (Fereol et al., 2005a). According to t-test, no significant difference was found between the virus statuses at 5% level.

Table 5. Regeneration of suspension culture function of virological status

Type of callus induction %	**original virus status**		*T test*
	Vrirus (-)	**Virus (+)**	
number of container of culture	**6**	**6**	
Mean Nb. of plantlets regenerated (/0.1ml SCV)	**109.1**	**102.1**	*0.43NS*

NS: no significant difference between the virus status
at 5% level by T test

4.6 Adapting to in Vivo Conditions

The *in vitro* rooted plantlets were successfully transplanted in the glass greenhouse - 95%.

Two to three months after transplanting in green house, the plants were large enough for virus testing or transplanting to the field in net house. The efficiency of regeneration ranged from 40 to 50%.

4.7 Virological investigations

Of 80 germinated shoots of garlic tested, 4 were found positive for OYDV, 3 positive to LYSV, and 1 positive to these both viruses. Somatic embrogenesis strategy through suspension culture was applied to this infected donor plant to test the possibility of eliminating OYDV and LYSV along the process of somatic embryogenesis. The scheme adopted is shown in Fig. 3. Somatic embryogenesis process was also applied to a virus free donor from plants grown in net house. Although explants source were originally infected, all of the issued materials, until now proved to be virus-free. No virus could be detected by ELISA in any sample of neither callus, nor suspension culture and plant regenerated from these tissue types. The plantlets were transferred to the green house, and will be tested again for the presence of OYDV and LYSV.

5 DISCUSSION

Some viruses can be effectively eliminated from infected plants owing to their mode of replication and their mechanism of movement within the plant. The most widely used method for virus elimination is meristem tip culture. This technique takes advantage of the fact that many viruses fail to invade the meristem region. Transfer of the meristem dome, to a culture medium and development into a plantlet may lead to the elimination of a virus. Successful elimination of *Sugarcane mosaic virus* and *Fiji disease virus* in sugarcane through apex or bud culture has been reported (Leu, 1972; Wagih et al., 1995). There are reports of the elimination of SCYLV using tissue culture (Chatenet et al., 2001; Fitch et al., 2001; Parmessur and Dookun, 2000). By using meristem culture, only a certain percentage of the plants developed from the excised meristems of infected plants are really virus free. The reasons for this includes the failure to eliminate viruses in explants. Consequently it is necessary to use combination of several techniques to produce virus-free garlic.

Callus proliferation may also lead to the elimination of viruses. Sugarcane yellow leaf virus (SCYLV) was eliminated from sugar cane, with 100% success when plantlets were derived from callus culture. A single callus subculture was found to be sufficient for eliminating SCYLV from infected plant material (Parmessur et al., 2002).

Somatic embryogenesis may get rid of some viruses in different species. These results were obtained with the grapevine (Goussard et al., 1991) and citrus (Carimi et al., 1994; De Pascale et al., 1994). Once the results in somatic embryogenesis are satisfactory, it is necessary to consider the efficiency of this method in the elimination of some viruses.

The results of the present study show that somatic embryogenesis has potentiality to be an effective method for OYDV and LYSV elimination, since the viruses were eradicated with 100% success. It was possible to eliminate OYDV and LYSV from infected plants by somatic embryogenesis process from leaf rolls. The uneven distribution of the virus in the different tissues of the shoot may account for its elimination. Lack of connection between the somatic embryos and the phloem limits movement of the virus or phytoplasma. Therefore plantlets regenerated from Embryogenic process are free from both pathogens. This is the first report using this method in garlic. A final testing 5 months after transplanting to soil might be

necessary to know if the plants remain virus-free. Latent infections must also be considered and consequently, repeated tested of established plants will be necessary to certify that the propagation material remain virus-free. The possible creation of variants by callus culture should also be taken into account.

However, the success of this method depends on an effective method of virus detection; a rigorous test is needed to ensure that the disease has been truly eliminated. The detection of the virus needs to be effective even at an early stage when the plantlets are still growing *in vitro.* At present, for viruses for which antisera are available, ELISA is the most widely used detection assay (Spiegel et al., 1993). Since it has been shown that the levels of the virus can fluctuate due to the seasonal variations, the detection with ELISA is not always possible (Scott et al., 1989; Torrance and Dolby, 1984). Consequently, methods based in polymerase chain reaction have started to be used (Spiegel et al., 1996). However, the prerequisite of knowing the viral sequences in order to synthesize specific primers limits the application of this approach to well-characterized viruses.

6 PERSPECTIVES

6.1 New approach to accelerate multiplication

The slow rate of conventional multiplication hampers the rapid dissemination of selected elite genotypes. In order to accelerate this process, ***in vitro*** **multiplication through axillary shoots** proliferation (Kahane et al., 1992) has been attempted and **succeed at AVRDC**. However, **new approach** to accelerating more garlic multiplication has been investigated **in this joint AVRDC-CIRAD project**. The method relies in developing embryogenic suspension cultures from somatic embryogenic calli. Appropriate and efficient methods for the induction of embryogenic callus, shoot development and suspension cultures have been developed to make the technique useful for seedlings production (Fereol et al., 2002; Fereol et al., 2005a; Fereol et al., 2005b). It would shorten the outside phase for base stock establishment, and would provide a larger quantity of material so that the phase of field multiplication could be limited to 2 cycles. **This change would** considerably **limit the risk**

of viral contamination during the cycles of field multiplication, and it would thus reduce plant production costs.

6.2 Cryo-preservation Germplasm management

Since garlic is **vegetatively propagated**, genetic resources are traditionally **maintained in the field collections**. This approach offers some advantages, because the accessions under this type of conservation can be readily accessed and observed, allowing detailed evaluation. However, field conservation **risks the build up of various viral, bacterial or fungal infections**, which threatens long term preservation. Other natural hazards such as drought, weather damage, vandalism, and human error may also undermine the collection. **Germplasm exchange** over long distances **may be limited** due to the risks of disease transfer through vegetative material. The **high cost of maintaining field genebanks** may also limit the number of accessions that can be managed. Such constraints inhibit the expansion of the collection to increase the diversity of the germplasm, and limit the possibilities of conducting systematic studies of diversity or crop evolution. Consequently, researchers are seeking **alternative preservation methods**, and **cryopreservation** offers significant advantages and opportunities to complement other germplasm conservation strategies (Engelmann, 2004).

Cryopreservation (liquid nitrogen, -196°C) has been **applied for storage of different plant species**, many of tropical origin (Engelmann and Takagi, 2000; Keller, 2002; Makowska et al., 1999; Niwata, 1995), providing **cost-effective** and **long-term** (if not indefinite), **stable conservation** of genetic resources. It has been feasible also in garlic (Baek et al., 2003; Keller et al., 2003; Keller et al., 2001; Kim et al., 2004). Various types of tissues may be subjected to cryopreservation: Seeds, shoot tips or buds are currently successfully conserved. **Somatic embryos or cell suspension cultures** might also prove useful subject tissues, once plant regeneration in all genotypes is possible and off-types are minimized. Coordinated with other techniques of germplasm management (field collections, seed archives, etc), cryopreservation of **a range of tissue types** can add flexibility, reduced costs and stability to the preservation and exploitation of genetic diversity (Sakai et al., 2003).

6.3 Cryotherapy

Cryo-therapy: In 1997, (Brison et al., 1997) demonstrated for the first time that **cryo-treatment** can not only be used for germplasm conservation but also **for virus eradication**. Cryopreservation resulted in 50% virus-free *in vitro* plants from plum shoots infected with plum pox virus. (Helliot et al., 2002) reported on **successful CMV eradication by cryopreservation** of highly proliferating meristems of banana (cv. Williams BSJ, ITC.0570, AAA). Thirty percent of the regenerated plants were found to be healthy by DAS-ELISA, and this eradication rate was confirmed after a 6-month acclimatisation of plants in the greenhouse. This technique might be investigated for garlic.

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Abstract

Garlic (*Allium sativum* L.) is threatened by diseases, including a number of important viral diseases which represent a constraint on garlic production and on germplasm movement from country to country. The latter is especially important for farmers who are waiting to benefit from pest- and disease-resistant varieties, either naturally occurring germplasm or improved varieties produced by breeding programmes. A significant number of potentially important and improved varieties from breeding programme are infected with viruses. Most of these materials are infected with OYDV and LYSV. AVRDC has therefore been very active in establishing a system for the safe international movement of garlic germplasm.

To make these accessions available, a programme of virus elimination was carried on in the Plant Virology Unit (Dr. Green). *In vitro* technique, such meristem tip cultures were used to produce virus-free plantings of different clones infected with OYDV and LYSV. The health status of regenerated material was checked on *in vitro* plants through ELISA. The putative virus-free material was then tested a second time after greenhouse acclimatisation.

The use of tissue culture was investigated as a means to eliminate both OYDV and LYSV from infected cultivars. An infected cultivar was induced to form Embryogenic callus *in vitro* using leaf rolls as explants, and then suspension cultures. After ten subcultures on one hand of the callus, and on the other hand of the suspension culture, this cultivar was successfully regenerated and tested for OYDV and LYSV. No pathogen could be detected in any regenerated plantlets. All the regenerated plants remained free from both OYDV and LYSV over a period of 3 months in the glasshouse.

Somatic embryos were obtained from young leaf rolls as explants. Forty 3-month-old plantlets originating from leaf rolls culture-derived somatic embryos, twenty from callus source, and twenty from suspension gave negative reactions when assayed by serology for the presence of the viruses. It was concluded that, similar to other species (e.g. grapevine, sugarcane), somatic embryogenesis opens new possibilities for the sanitary improvement of garlic.
Key words: somatic embryogenesis, OYDV, LYSV
Abbreviations: 2,4-D, dichlorophenoxy acetic acid; BAP, 6-benzyl aminopurine; N6, salts of Chu et al. (1975) basal medium; SCV, Sediment Cell Volume; IAA, indol acetic acid; NAA, naphthalene acetic acid;

8 Annexe1. Stock Solutions of media used for garlic embryogenesis

Macro nutrients (mg)	(N6)		(SHm)			
Concentration	Final Sol	X 20	Final Sol	X 20		
Quantity stock solution per liter of final solution		50 ml		50 ml		
Quantity of stock Solution to do		**1000 ml**		**1000 ml**		
-KNO3	2850	57 g	2500	50g		
-(NH4)2SO4	463	9.26g				
-NH4H2PO4			300	6g		
-KH2PO4, 2 H2O	400	8g				
-CaCl2, 2H2O	166	3.32g	440	8.8g		
-MgSO4, 7H2O	185	3.7g	400	8g		
Micro nutrients (mg)	**(MS)**					
Concentration	Final Sol	X 1000				
Quantity mother Sol for 1 l of final Sol		1 ml				
Quantity of mother Sol to do		**1000 ml**				
- H3BO3	6,2mg	6200				
- KI	0,83	830				
- MnSo4, 4H2O	16,7	1670				
- ZnSo4, 7H20	8,6	8600				
- CoCl2, 6H2O	0,025	25				
- CuSo4, 5H2O	0,025	25				
- Na2MoO4, 2H2O	0, 25	250				
Vitamins (mg)	**L2**		**L9**		**SHm**	
Concentration	Final Sol	X 200	Final sol	X 50	Final sol	X 200
Quantity mother Sol for1 liter of final Sol		5 ml		20 ml		5 ml
Quantity mother Sol to do		**100 ml**		**1000 ml**		**100 ml**
- Glycin (mg)	20	400	2	100		
- Nic ac (mg)			1	50	5	100
- pyridoxine (mg)			1	50	0.5	10
- Thiamine HCl (mg)	40	800	10	500	5	100
- serin			10	500		
- cystein (mg)	20	400	20	1000		

Fe- EDTA (mg)			
Concentration	Final	X 100	Pour Na2 EDTA heat, in FeSO4 heat, progessively to avoid precipitation
Quantity mother Sol for 1 l of final Sol		10 ml	
Quantity mother Sol to do		**1000 m**l	
- FeSO4, 7H2O	27.8	2.785 g in 500 ml	
- Na2 EDTA	37.3	3.725 g in 500 ml H20,	
Organic substances			
Concentration	Final Sol	X 200	
Quantity mother Sol for 1 l of final Sol		5 ml	
Quantity mother Sol to do		**500 m**l	
- Malt extract	400 mg	40 g	
- Casein Hydrolysat	100	10 g	
- Myo-Inositol	400	40g	
Growth regulator (mg)			
- 2.4.D	1mg = 10ml	50mg+ 2,5ml alcohol 90° + 500ml H2O	
-NAA	1mg = 10 ml	50mg+ 1 tablet KOH+ few H2O: dissolve, then complete500ml H2O	
- IAA	1mg = 10 ml	50mg+ 2,5ml alcohol 90° + 500ml H2O	
- BAP	1mg = 10 ml	50mg+ 1 tablet KOH+ 500ml H2O	
- Kinetin	1mg = 10 ml	50mg+ 1 tablet KOH+ 500ml H2O	

9 Annexe 2.

Double Antibody Sandwich ELISA (DAS-ELISA)

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Before opening the tubes containing coating antibody (IgG) and conjugate (IgG-AP).
Centrifuge!! to collect the content at the bottom of the tube

1. Dilute purified IgG in coating buffer (recommended dilution see delivery note and tube).
   i.e. for 100 tests: 20 µl in 20 ml buffer at a recommended dilution of 1:1000; 40 µl in 20 ml buffer at a recommended dilution of 1:500. Or at equal ratios for other volumes.
   Add 200 µl to each well of a microtitre plate.
2. Incubate at 37 °C for 2-4 h.
3. Wash plate with PBS-Tween using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.
4. Add 200 µl aliquots of the test sample (extracted in sample extraction buffer) to duplicate wells.
5. Incubate overnight at 4 °C.
6. Wash three times as in step 3.
7. Add 200 µl anti-virus conjugate, recommended dilution is given in the delivery note, in conjugate buffer to each well.
8. Incubate at 37 °C for 4 hours.
9. Wash three times as in step 3.
10. Add 200 µl aliquots of freshly prepared substrate (10 mg p-nitrophenyl phosphate [Sigma 104-105] dissolved in 10 ml of substrate buffer) to each well. Incubate at room temperature for 30-60 min, or as long as necessary to obtain clear reactions.
11. Assess results by:
    a) Visual observation
    b) Spectrophotometric measurement of absorbance at 405 nm

Reference
Clark, M. F. and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.

Buffers used in ELISA

1. *Coating buffer (pH 9.6)*
   1.59 g sodium carbonate (Na_2CO_3)
   2.93 g sodium bicarbonate ($NaHCO_3$)
   0.20 g sodium azide (NaN_3)
   Dissolve in 900 ml H_2O, adjust pH to 9.6 with HCl and make up to 1 l.

2. *PBS (pH 7.4) phosphate buffer saline*
   8.0 g sodium chloride (NaCl)
   0.2 g monobasic potassium phosphate (KH_2PO_4)
   1.15 g dibasic sodium phosphate (Na_2HPO_4)
   0.2 g potassium chloride (KCl)
   0.2 g sodium azide (NaN_3)
   Dissolve in 900 ml H_2O, adjust pH to 7.4 with NaOH or HCl and make up to 1 l.

3. *PBS-Tween (PBST)*
PBS + 0.5 ml Tween 20 per liter

4. *Sample extraction buffer (pH 7.4)*
PBST + 2% PVP (Serva PVP-15 polyvinyl pyrrolidone)

5. *Conjugate buffer*
PBST + 2% PVP + 0.2% egg albumin (Sigma A-5253)

5. *Substrate buffer*
97 ml diethanolamine
600 ml H_2O
0.2 g sodium azide (NaN_3)
Adjust to pH 9.8 with HCl and make up to 1 liter with H_2O

Buffers can be stored at 4 ° C for at least 2 months. Warm to room temperature before use.

ELISA Troubleshooting

1. *No color development*

a) Did you omit any steps?
b) Did you use correct buffer for each step?
c) Is your enzyme OK? Serum OK?
d) Is your positive control homologous to antiserum (IgG)?

Recommendations - Do a titration plate. Use reliable positive control in each plate. Pretest enzyme conjugate on substrate. Check purified IgG for antigen-specific IgG.

2. *Nonspecific color development*

a) If in edge wells only:
- Make sure the humidity in the incubator is sufficiently high.
- If this does not help, don't use edge or border wells, fill with buffer only.

b) If in whole plate:
- incomplete washing
- old substrate
- error in loading sequence
- conjugate still contains glutaraldehyde: dialyse again

Recommendations - Use reliable negative control in each plate. Absorb antiserum against healthy plant extract. Use fresh substrate and check for spontaneous color change. Cover plates. Check pH of the buffers used.

c) Some wells with inconsistent or unexpected reactions
- incomplete washing
- error in loading test antigens
- spillage between wells

Recommendations - Use extra wash step, handle plates carefully with lids on, use predetermined loading pattern before loading. Blot top of plate after rinsing.

3. *Color development very rapid; some color in healthy samples*

a) Enzyme conjugate concentration too high
b) Substrate concentration too high

Recommendations - Use enzyme conjugate and substrate concentrations that will give $OD_{405\ nm}$ of about 1.0 in 30 to 60 min with good antigen source.

TRAINING COURSE ON "THE APPLICATION OF BIO-MOLECULAR TOOLS FOR BREEDING"
IN CIRAD, FRANCE

for Ms Cherng Shin-jiun (Vicky)
from September, 1st to October, 29th 2005
Responsible : Dr Marie France Duval

This action is situated in the objective to prepare to bring more tools in the onion breeding program.

In the framework of the MOU between NSC (ROC) and INRA-CIRAD (France), according to the MOU between CIRAD and AVRDC, and following the general agreement to strengthen their scientific collaboration, CIRAD Department Flhor was pleased to confirm the invitation of an agent of AVRDC (Ms Cherng Shin-jiun, Vicky) to a training on "The application of bio-molecular tools for breeding".

CIRAD offered to pay the following costs : boarding and training fees.
NSC paid the travel air ticket.

PROGRAM PROPOSED:

Practical training :

1. Basics on molecular markers and their use. Dr Jean-Louis Noyer
2. DNA extraction and purification
3. SSR (Single Sequence Repeat) markers
4. AFLP (Amplified Fragment Length Polymorphism) markers
5. Methodologies used for data scoring and analysis
6. Fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH)

SSR (Single Sequence Repeat) markers

Discussions with the Quality and Safety lab staff

Meeting with staffs of CIRAD:

1. Dr. Jean –Marc Lacape, UMR-PIA CIRAD

Marker assistant selection on cotton quality

2. Dr. Norbert Billotte, UMR-PIA CIRAD
   Marker assistant selection and maping on palm tree
3. Dr. Jean-Louis Noyer, UMR-PIA CIRAD
   Chief of the team on Diversity study by molecular marker on cultivated plants
4. Technetium Magey Rodier-Goud, UMR-PIA
   Cytogenetic platform on banana, rubber tree etc.
5. Dr. Marie-France Duval, UPR-MV CIRAD
   SSR on pineapple and mongo and its apply to other crops
6. Remi Kahane, Fruit and Horticultural Crops Deportment
   Garlic research in CIRAD

Meeting with visitors:

1. Dr. E.R.J Keller, Gene bank IPK, Germany
   Introduction the gene bank of Institute of plant genetic and crop plant research
   In vitro storage and cryopreservation of Allium crops.
2. Dr. Chris Kik, CGN , Netherlands
   Seminar on introduction of the Center for Genetic Resources (CGN)
   Garlic and Health project
   Allium research at Plant Research International

Field trip to onion production area in Cevennes

TRAINING COURSE ON CRYO-PRESERVATION IN CROP PLANTS : TECHNICAL ASPECTS, CELL BIOLOGY AND PERSPECTIVES IN CIRAD, FRANCE

for Ms Chang, Ching-huan (Jessica)
from October, 15th to December, 10th 2005
Responsible CIRAD persons : Dr R. Kahane/ Dr F. Cote and Dr P. Ollitrault

This action is situated in the objective to prepare to set up the technique of cryo-preservation in the management of germplasm conservation of AVRDC.

In the framework of the MOU between NSC (ROC) and INRA-CIRAD (France), according to the MOU between CIRAD and AVRDC, and following the general agreement to strengthen their scientific collaboration, CIRAD Department Flhor was pleased to confirm the invitation of an agent of AVRDC (Mrs Chang Ching-huan, Jessica) to a training on "The application of *Cryo-preservation in crop plants : technical aspects, cell biology and perspectives*".

CIRAD offered to pay the following costs: boarding and training fees.
NSC paid the travel air ticket.

PROGRAM PROPOSED:

Meeting with Cirad staff

visit of the laboratories "Banana" CIRAD and "Coffee" IRD

Discussions about cryo-preservation technology. Régis Domergue

Cryo-preservation techniques and their use. Régis Domergue

Somatic embryogenesis and embryo rescue in a breeding programme. D. Dambier

Protoplasts fusion and it use in Citrus breeding programme. D. Dambier, P. Ollitrault

Bibliography and visit of other laboratories (Vitropic, Biotrop). R. Kahane

Discussions with the Quality and Safety lab staff.

During the training periods has been organized various meetings with researchers involved in cellular biology programmes and the applied projects (use in breeding, in genetic resources management, etc…), according to their availability.

PROPOSAL: ABSTRACT

CRYOPRESERVATION FOR GERMPLASM CONSERVATION AND SUPPLY OF DISEASE FREE SEEDLINGS OF VEGETATIVELY PROPAGATED VEGETABLES

Dr. Leonidas FEREOL
Dr. Liwayway M. ENGLE
Dr. Sylvia GREEN
Dr. Haeng-Hoon KIM

(DRAFT, December 2005)

Proposal to submit to DED, GTZ, IPGRI …

1 General objectives and expected achievements

1.1 Strategic objectives addressed

The **goal** of this project is to advance the **development of new collected *Allium* and improved *Allium* varieties** through the development of optimized genetic resources systems, thereby **ensuring the sustainable cultivation of *Allium* in different regions of the tropics**. In order to address **economic, nutritional and healthy shortfalls** in the developing world, the project brings together major scientific expertise in plant breeding, biotechnology, and germplasm management. New approaches to vegetable pathology, variety development, conservation, multiplication and distribution will be developed. These approaches might serve as a **model for** development strategies in **other vegetable crops**.

This proposal presents the rationale, scientific background, technical details, and workplan to achieve the following objectives:

Objective 1. Improvement of commercial vegetable crops varieties adapted specifically **to tropical regions** through multidisciplinary approaches, combining pathology, physiology, breeding and biotechnology expertise.

Objective 2. Development and efficient **use of cryopreservation for germplasm management** and cryo-therapy against some pathogens.

1.2 Expected achievements

The objectives of the project will be achieved by combining techniques with innovative biotechnical approaches, including: *in vitro* clean up against diseases, embryogenesis and cell suspension culture techniques, and cryopreservation strategies. We expect to reach the following achievements:

- **Set up of cryopreservation technique to AVRDC germplasm conservation.**
- **Obtaining new way for virus elimination such as therapy through tissue culture and cryo-therapy.**
- Speed up the distribution of new cultivars by new propagation processes.

2 Exploitation and dissemination results

Introduction of **improved vegetables materials** particularly garlic and **other clonally propagated vegetable crops** for the tropics will **strengthen the international competition force of tropical vegetables growers** by giving added value to their product, also by realistic sustained health claims concerning these species. The programme may lead to the **development of new potent phyto-pharmaceuticals.**

The **benefits** of this project **to the human quality of life** and management of living resources programme are manifold:

- Contribute to **comprehensive overview of the world gene-pool of *Allium*** and other vegetable crops.

- The **availability of a large documented collection of *Allium*** and other clonally propagated vegetable crops particularly for tropical regions represents a unique starting point for future agronomic research in these species.

- **Efficient cryopreservation protocols** shall be applied **in international and national genebanks**

Capacity of the partnership for industrial exploitation

Breeding companies, farmer's groups and **pharmaceutical industries** may be **interested in this project**.

Plant Cell Report (2005), 24: 319-325

PUBLICATION 1: ABSTRACT

ESTABLISHMENT OF EMBRYOGENIC CELL SUSPENSION CULTURES OF GARLIC (ALLIUM SATIVUM L.), PLANT REGENERATION AND BIOCHEMICAL ANALYSES

L. Fereol [1,2], V. Chovelon, S. Causse, D. Triaire , I. Arnault, J. Auger and R. Kahane

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Abstract

Embryogenic cell suspension cultures of garlic were initiated in liquid medium, from friable embryogenic tissue. The optimal parameters were for culture maintenance: 1) an initial cell density of 1-4% (vol/vol); 2) medium renewal every 14 days and subculturing every 28 days; and 3) a low 2,4-D concentration (0.1-0.3 mg/l). Cultures regenerated throughout 14 months. The cell suspension cultures differentiated embryos after transfer to a semi-solid embryo induction medium. Histological studies confirmed and characterised the embryogenic nature of the process. Forty percent of these embryos were converted into plantlets, which produced micro bulbs *in vitro*. The composition of sulphur compounds of micro bulbs from cell suspension embryo derived plantlets differed slightly from those produced by *in vitro* shoot proliferation derived plantlets, but after two cycles of multiplication in the field these difference was not maintained.

Key words: somatic embryogenesis, *histology,sulphur compounds*

Acta Horticulturae, (2005) 688 : 65-74

PUBLICATION 2: ABSTRACT

EMBRYOGENIC CELL SUSPENSION CULTURES OF GARLIC (ALLIUM SATIVUM L.) AS METHOD FOR MASS PROPAGATION AND POTENTIAL MATERIAL FOR GENETIC IMPROVEMENT

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Keywords : somatic embryogenesis, histology, plant regeneration, multiplication rate

Abstract

Embryogenic calluses were induced from young leaf explants of garlic (Allium sativum L). Four cultivars, Rouge de la Réunion, Messidrome, Morasol and Printanor have been successfully tested. These calluses expressed up to 90% of embryogenic calluses differentiating globular somatic embryos after 2 months on N6 modified medium supplemented with 2,4-D (0.1 mg l-1) and kinetin (0.5 mg l-1). Embryogenic calluses were used to establish cell suspension cultures of the above-mentioned cultivars. Friable calluses were induced from compact ones, and could give rise the production of cell suspension cultures composed of small aggregates of embryogenic cells. These suspension cultures were maintained in liquid medium based on N6 modified macro-nutrients and supplemented with 2,4-D/benzyladenine (0.3 mg l-1/0.1mg l-1). The packed cell volume (PCV) of the suspension cultures increase 2-fold in a 2-week period. These cell suspension cultures led to successful regeneration of mature embryos and their conversion into plantlets. Optimal embryo regeneration efficiency was obtained after plating on semi-solid medium base on N6 macro-nutrients and a balance in 2,4-D/Kinetin (0.1mg l-1/0.5mg l-1). A large number of somatic embryos (potentially 8 x 109 to 1011) could be produced per year for each cultivar. The conversion into plantlet was approximately 50%. Plants were successfully acclimatised in greenhouse. Histological analyses were performed along the suspension cultures and regeneration process, and helped for establishing the sequence of culture media. The somatic embryogenic nature was confirmed by single cell origin and polar development of the regenerants. This protocol was used in a goal of mass propagation of garlic plants true to the original type. It would be a key tool for biotechnologies in genetic improvement of garlic.

Production de masse de plants sains d'ail par embryogenèse somatique

L. Fereol[1], V. Chovelon[2], S. Causse[1], M.L. Kalumvueziko[3], R. Kahane[3]

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*Le contrôle des maladies virales causées par deux potyvirus, Onion Yellow Dwarf (OYDV) et Leek Yellow Stripe (LYSV), représente une importante contrainte pour la culture de l'ail (*Allium sativum *L.), d'où la nécessité de mettre en place un système de production de plants débarrassés de ces virus. Dans cette optique, la production de masse de plants sains par voie de suspension cellulaire embryogène est très prometteuse. Les virus OYDV et LYSV n'étant pas transmissibles par embryogenèse zygotique (Chovelon, comm. pers.), nous avons testé l'efficacité de l'embryogenèse somatique vis-à-vis de cette non-transmission.*

Matériel et méthodes

Matériels

Quatre variétés d'ail appartenant à différents groupes physiologiques ont été utilisées : 'Morasol', 'Messidrome', 'Printanor' et 'Rouge Réunion'.

Méthodes

1. Des cals nodulaires puis friables, embryogènes, ont été obtenus à partir d'explants de jeunes feuilles d'ail [1].
2. Ces cals cultivés en milieu liquide ont induit des cultures cellulaires en suspension [2].
3. L'étalement de cette suspension sur un milieu semi-solide a permis de régénérer des embryons somatiques qui ont été ensuite convertis en plantules [2].

Culture cellulaire en milieu liquide obtenue à partir de cals embryogènes issus d'explants foliaires de *A. sativum*.

Régénération en masse d'embryons somatiques induits par culture cellulaire (*A. sativum*).

Résultats

Régénération et conversion

Après étalement, la culture de cellules en suspension a produit de nombreux embryons somatiques en 8 semaines. Ceux-ci ont présenté une structure bipolaire ayant ses apex caulinaire et racinaire reliés par des vaisseaux vasculaires. Le procédé a été appliqué avec succès à tous les cultivars expérimentés. À partir de 1 ml de masse cellulaire, 3000 à 7000 embryons matures peuvent être obtenus. Le pourcentage de conversion en plantules a été de 36 à 51 %.

Régénération d'embryons par culture cellulaire et conversion en plantules (*Allium sativum* L.). Les différences sont significatives à $p < 0,0001$ selon le test F de l'analyse de variance.

Cultivar	Nombre d'embryons globulaires par ml de masse cellulaire (V1)	Nombre d'embryons matures par ml de masse cellulaire (V2)	Nombre d'embryons convertis en plantules (V3)	% d'embryons convertis en plantules (V3/V2)
Rouge Réunion	6850 b	4050 b	2100 b	51,82 a
Messidrome	4835 c	3008 c	1083 c	36,03 c
Morasol	13500 a	7833 a	3608 a	46,12 b
Printanor	5250 c	4392 b	1817 b	41,25 bc

Dans une même colonne, les lettres indiquent les groupes homogènes selon le test Khi-2 ou test F ($p < 0,05$).

Taux comparatif de multiplication

Selon les cultivars, le taux de multiplication a varié de 10^9 à 27 x 10^9 en 16 mois. Ce taux peut être plus de 100 fois supérieur à celui obtenu avec les autres méthodes de multiplication. Un à deux cycles de propagation traditionnelle sont ensuite nécessaires pour fournir des plants de qualité standard.

Quantification estimée par étape pour la production de masse de plants à partir d'une culture cellulaire d'*Allium sativum* L. (5 explants mis en culture pour 1 cayeux utilisé par cultivar testé).

Cultivar	Nombre de cals friables embryogènes (en 6 mois)	Taux mensuel d'amplification de la suspension (de 8 à 12 mois)	Nombre d'embryons régénérés par ml de masse cellulaire (en 14 mois)	Taux (%) de conversion des embryons en plantules (en 16 mois)	Nombre estimé de plantules pouvant être obtenues en 16 mois
Rouge Réunion	5 x 0,52 x 4^{16}	x 6,0	4050	50	$\approx 6 \times 10^{16}$
Messidrome	5 x 0,56 x 4^{16}	x 7,1	3010	36	$\approx 20 \times 10^{16}$
Morasol	5 x 0,63 x 4^{16}	x 6,5	7500	48	$\approx 27 \times 10^{16}$
Printanor	5 x 0,45 x 4^{16}	x 4,7	4390	41	$\approx 10^{16}$

Conclusions

La culture cellulaire en suspension présente une très grande efficacité pour la production en masse de plants sains d'ail ; elle peut ainsi constituer l'un des maillons de la lutte intégrée. De plus, la méthode exposée pourrait permettre de filtrer certaines maladies virales.

Références

[1] Fereol L., Chovelon V., Causse S., Michaux-Ferriere N., Kahane R. (2002). Evidence of a somatic embryogenesis process for plant regeneration in garlic (*Allium sativum* L.). Plant Cell Rep. 21 : 197-203.

[2] Fereol L., Chovelon V., Causse S., Triaire D., Arnault I., Auger J., Kahane R. (2005). Establishment of embryogenic cell suspension cultures of garlic (*Allium sativum* L.), plant regeneration and biochemical analyses. Plant Cell Rep. (accepté pour 2005).

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