HYBRIDIZATION IN VETCH

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HYBRIDIZATION IN VETCH

By

Richard L. Cooper

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Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements

MASTER OF SCIENCE

Department of Farm Crops

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AN ABSTRACT

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ABSTRACT

Some 24 species of *Vicia* were assembled and grown in the greenhouse. Many interspecific crosses were made, with special emphasis on crosses between *V. villosa*, *V. dasycarpa*, *V. sativa*, *V. pannonica* and *V. atropurpurea*. In addition several intraspecific crosses were made between 130 entries of *V. sativa*.

No hybrid seed was obtained from crosses of the five major species. Seed was obtained, though, in crosses of *V. calcarata* with *V. sativa*. *V. calcarata* is listed as a distinct species by Darlington and Wylie (9). However, the strong morphological similarity of *V. calcarata* and *V. sativa* suggests a possible synonymy of these two species.

Other successful crosses were between *V. cornigera* and *V. sativa*, and between three purple flowered species, *V. onobrychoides*, *V. bengalensis*, and *V. atropurpurea*. However, because of the strong morphological similarities of *V. cornigera* and *V. sativa*, it is suggested that *V. cornigera* is a selection of *V. sativa* rather than a distinct species. Similarly, the high cross compatibility and morphological similarity of the purple flowered species suggests that *V. onobrychoides* and *V. bengalensis* are selections of *V. atropurpurea*. *V. cornigera*, *V. onobrychoides* and *V. bengalensis* were not listed by Darlington and Wylie (9).

Intraspecific crosses in V. sativa were quite fertile, producing 58 different hybrid combinations. These F_1 hybrids, together with their parents were planted in the East Lansing nursery in the summer of 1958. In several cases, the F_1 hybrid exhibited greater vigor and set more seed than either parent.

Preliminary experiments in embryo culture were conducted in an effort to find nutrient media satisfactory for growing small immature embryos of vetch. Various basal media were used, supplemented with varying amounts of sucrose, vitamins and the amino acids, asparagine and glutamic acid. Coconut milk and casein hydrolysate media were also tried.

Nearly mature V. villosa embryos were successfully grown on Rijven's basal medium supplemented with asparagine, 200, 800, and 1600 mg/l, and sucrose 2 and 12 percent. Large V. atropurpurea embryos germinated and grew in Rijven's basal medium supplemented with asparagine and glutamic acid, each at 200 mg/l, and sucrose 2 and 12 percent. All attempts to grow smaller embryos were unsuccessful.

Vetch seeds, either presoaked in water or dry, were treated with 0.2, 0.4 and 0.8 percent aqueous solutions of colchicine in petri dishes. If presoaked, the seeds were treated for six and 24 hours duration, and if not presoaked, for ten and 24 hours. In a side experiment, 250 and 500 ppm of gibberellin were added to the water or the colchicine solutions.

One plant, V. villosa, was obtained that exhibited tetraploid sectors. This plant was obtained from seed treated directly in 0.8 percent colchicine solution for 24 hours.

Using Nygren's technique, some one hundred V. atropurpurea plants were treated with nitrous oxide gas. The plants were put in a gas chamber under pressures of 50, 75, and 100 pounds for durations ranging from four hours to 16 hours. Plants were treated at 10, 13½, 17, 20½ and 24 hours after pollination in an effort to bracket a time that would place a plant under treatment when the first mitotic division of the newly formed zyogote occured.

No seed was set in treatments more severe than 75 pounds for ten hours. Plants survived treatments as high as 100 pounds for ten hours. If duration of the treatment exceeded ten hours the only seed produced was at 50 pounds pressure. No seed was produced in the 16 hour treatments.

Time did not permit cytological examination of root tips from all seeds produced, but in a sampling from each treatment, no polyploid cells were observed.

References

1. Nygren A. "Polyploids in Melandrium Produced by Nitrous Oxide." Hereditas, 41:287-290, 1955.

2. Rijven, A. H. G. C. "Glutamine and Asparagine as Nitrogen Sources for Growth of Plant Embryos In Vitro: a Comparative Study of Twelve Species." Australian Journal of Biological Sciences, 9:511-527, 1956.

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INTRODUCTION

In 1953, two and one half to three million pounds of hairy vetch seed were produced in Michigan (22). Most of this seed was shipped to the Cotton Belt States in the South where vetch is important as a winter cover and green-manure crop. Four years later, in 1956, only a few thousand pounds of vetch seed were produced in Michigan.

The major reason for this sharp decline in vetch seed production has been the introduction and spread of the vetch bruchid throughout the state of Michigan.

The bruchid is highly specific, attacking only two species of vetch, V. villosa and V. dasycarpa. The larvae of this insect feed in the developing seed of these vetch species, destroying the germination.

There are many species of vetch resistant to bruchid infestation including the economically important vetches V. sativa, V. pannonica, and V. atropurpurea.

Therefore in the summer of 1957, some 24 different species of vetch were assembled from various plant introduction stations and foreign countries. These plants were grown in the greenhouse and many different interspecific crosses were attempted, with special emphasis on crosses between major economic species.

However, through personal communication with other investigators working with vetch it was indicated that a high degree of species incompatibility exists in the genus Vicia. Thus, an effort was made to find techniques which could be used for overcoming these species incompatibilities.

Several investigators have been successful in overcoming interspecies barriers in various genera by the use of embryo culture. Therefore experiments were set up in effort to obtain a culture medium that would satisfactorily grow vetch embryos.

Another successful tool of the plant breeder in overcoming species incompatibilities has been polyploidy. Experiments were run with colchicine and nitrous oxide in effort to develop techniques which would readily produce polyploid vetches.

LITERATURE REVIEW

Of the 150 species of Vicia (vetch) distributed throughout the world, only a few are of economic importance in the United States. Vetches are limited in their use because of disease and insect susceptibility and lack of winter hardiness. Attempts to combine desirable characteristics of different species have been unsuccessful due to species incompatibility. Though embryo culture and polyploid induction have been used successfully in overcoming interspecific barriers in other genera, these techniques have not been utilized in a breeding program in vetch.

The Vicia species of major importance in the United States are: Vicia villosa, hairy vetch; V. sativa, common vetch; V. atropurpurea, purple vetch; and V. pannonica, Hungarian vetch, as listed by McKee (21).

According to Henson and Schoth (13), diseases of major importance on vetch are: anthracnose, false anthracnose, black stem, grey mold leaf spot, and various root rots. Most insect pests that attack other legumes also attack vetch. Of particular importance is the green pea aphid.

The vetch bruchid is a serious pest in areas where seed of V. villosa is produced. According to Nelson and Janes (22), the vetch bruchid was first discovered in New Jersey in 1930 and had spread over the entire country by 1956.

Pinckney and Stitt (28), in 1941 reported that in species tests for susceptibility to bruchid injury, V. villosa and V. dasycarpa showed considerable damage while V. atropurpurea showed only slight infestation. All other 14 species of vetch tested showed no damage, including V. sativa and V. pannonica, even though a few eggs were laid on the pods of these species. Weimer and Bissell (42), in 1942 reported similar results in testing resistance to bruchid with the exception that they found no damage on V. atropurpurea.

Through personal communication with other investigators maintaining a vetch breeding program, it was indicated that all efforts to transfer bruchid resistance to V. villosa by interspecific crosses have been unsuccessful. Species barriers prevented the formation of hybrid seed.

These species barriers are particularly strong in the genus Vicia, and only a few interspecific crosses have been reported in literature. Yamamoto (46), in 1955 reported a successful cross between V. sativa and V. tetrasperma and later in 1956, he reported obtaining hybrid seed between V. sativa and V. angustifolia (47). Yitzchake (49) also obtained hybrid seed in crosses between V. sativa and V. angustifolia. Progent (29), reported the development and use of Cerdagne vetch, a hybrid between V. villosa and V. cracca. Of interest, is an intergeneric cross between Lens esculenta and V. sativa, reported by Buchinger (6). (Fig. 1-12)

Embryo Culture

Several investigators have been able to overcome interspecific barriers by use of embryo culture. Hannig (12), in 1904 is recognized as the first investigator to excise and culture immature embryos successfully, while Knudson, L. (17), in 1916 is credited with establishing much of the basis for culturing embryos and seeds in vitro in his work studying the influence of maltose on growth of corn embryos.

Laibach (18), in 1929 realized the possibility of obtaining hybrids from interspecific crosses which otherwise produced shrivelled, inviable seeds by excising immature hybrid embryos. By means of embryo culture he was able to nurse the hybrid embryos of Linum austriacum and Linum perenne to maturity. In 1942, Skirm (37), emphasized the use of embryo culture methods as an aid to plant breeding. He successfully cultured interspecific hybrid embryos in Prunus and Lillium, which when not excised did not fully develop due to embryo starvation.

Keim (16), in 1952 reported success in growing excised embryos of several species of Trifolium and Lotus in Randolph's agar medium. (31) By using embryo culture he was able to obtain interspecific hybrids in Trifolium. He also developed an embryo culture technique wherein he was able to excise and culture embryos with a contamination of less than 1 percent.

Since Hannig's first embryo culture in 1904, where he used Tollen's nutrient medium, many new and more elaborate ones have been developed taking into consideration the latest findings on the nutritional requirements of embryos. Because of numerous media it was decided to limit the discussion to the more pertinent work.

Basal media used in the present experiments were those developed by Randolph (31), Rappaport (32), White (44), and Rijven (34). Based on reports by numerous investigators, these basal media were then supplemented with: trace elements, sucrose, vitamins, amino acids and different organic substances.

White (43), in 1951 demonstrated that excised tomato roots required iron, copper and molybdenum and probably zinc, manganese, boron and iodine. Nitsch (24) developed a trace element solution that he added to his nutrient media. Similarly, Rijven (34) included the trace elements in his medium. Other workers such as Rappaport (32) limited their media to only a few of the trace elements.

In the earlier media, iron salts used for an iron source had a tendency to precipitate in prolonged cultures rendering the iron unavailable. Rappaport (32) replaced the relatively unstable iron sulphate in Randloph's culture by a more stable iron complex, iron citrate. The use of iron citrate has in general been adopted by most investigators.

Carbohydrates

A carbohydrate source in the nutrient media serves two purposes, one as an energy source and another as an osmotic agent. Rappaport (32) in a review of literature on embryo culture, 1952, found that in general investigators obtained best results by the use of sucrose as the carbohydrate source in their nutrient media. However, embryos of some plant species seem to prefer fructose, dextrose or other sugars in the nutrient media.

Tukey (40), in 1938 reported that the sugar requirement of excised embryos depended on the stage at which the embryos were excised. Rappaport found, that in Datura, very young embryos in the "heart" and "preheart" stages, grew best on media containing up to 8 percent sucrose. The older the embryos at time of excision, the lower the optimal sucrose concentration becomes until it reaches a level of about 0.5 percent with "late torpedo" stage.

Sanders (35), in 1950 found that growth in Datura stramonium embryos with 4 percent sucrose was 42 times that obtained with 0.5 percent, whereas over the same range, growth of three other Datura species increased only 1.2 to 2.7 times. Thus, even species within the same genus may react differently to different sugar concentrations. Honma (15), in 1955, by changing the sucrose concentration in his nutrient medium from 4 percent down to the 0 percent level was able to obtain interspecific hybrids in Phaseolus. Previous attempts to grow these interspecific hybrids had been unsuccessful.

Amino Acids

According to Rappaport (32) in his survey of embryo culturing techniques, many investigations with plant tissue fragments, organs, and embryos have shown that they can assimilate inorganic nitrogen. In general, organic nitrogenous compounds have been assumed to be inadequate nitrogen sources or even toxic when used in synthetic media. However, Sanders and Burkholder (35), in 1948 reported that the addition of a mixture of 20 amino acids resulted in notable growth of young Datura embryos.

Rijven (33), in 1952 in experiments with Capsella embryos, grown by a sitting drop technique, obtained stimulation in growth by the addition of glutamine and asparagine to his nutrient medium. In later experiments, 1956, he was able to detect beneficial effects in the growth of embryos from eight different orders of angiosperms by adding 400 mg/l each of glutamine and asparagine to the basal medium (34).

Glutamine was beneficial in every species tested whereas asparagine varied in effects, being inhibitory in some species in the order Rhoeadales. In a legume, Medicago orbicularis, asparagine was found to be progressively more beneficial at concentrations up to 2000 mg/l.

Glutamine was shown to enhance embryonic growth considerably more than asparagine in all cases. This was

ascribed to the fact that glutamic acid and glutamine have important primary roles in nitrogen metabolism, whereas asparagine is of lesser importance.

Vitamins

With the development of embryo culture techniques, it soon became apparent that some accessory growth factors were necessary in addition to inorganic salts and a carbohydrate source. White (44) was one of the first workers to add a vitamin solution to his nutrient media. This was later modified by La Rue (19), by taking 2.5 times the concentration of the vitamins and adding calcium pantothenate. Bonner et al. (2, 3, and 4), was able to demonstrate that nicotinic acid, ascorbic acid, and thiamine stimulated growth of excised pea embryos.

Van Overbeek, Conklin and Blakeslee (41), in 1942 reported that the addition of an arbitrary mixture of glycine, thiamine, ascorbic acid, pantothenic acid, nicotinic acid, vitamin B_6, adenine, and succinic acid proved effective in promoting growth of Capsella bursa pastoris embryos in the torpedo stage. They had previously failed to obtain growth at this early stage without the added vitamin solution.

Organic Media

Van Overbeek et al. (41), in 1942 using autoclaved coconut milk observed an unorganized growth of Datura embryos.

With unheated coconut milk however, they were able to obtain normal growth of very young embryos. Chang (8), in 1957 reported success in growing barley embryos, 0.43 mm in length, by the use of nine parts coconut milk to one part White's medium.

Sanders and Burkholder (36), in 1950 succeeded in growing very young Datura embryos by adding casein hydrolysate to their medium. Rappaport (32) reported similar success using casein hydrolysate.

Polyploid Induction

There are several techniques for inducing polyploidy in plants. Colchicine is by far the most frequently used of these techniques. Nitrous oxide treatment has the advantage in that the zygote can be treated during the first mitotic division. In this way a completely doubled plant can be obtained.

Colchicine

Blakeslee and Avery (1) were among the first investigators to utilize colchicine in producing polyploids in plants. Working with Datura, they were able to obtain from 45 to 65 percent polyploids by soaking pregerminated seeds in colchicine solutions varying from 0.2 to 1.6 percent concentrations for a period of ten days. Later experiments, using 0.4 percent concentrations, for one, two and four days he obtained as high as 100 percent polyploidy in some treatments.

Brewbaker (5), working with Trifolium species was able to obtain many polyploids by the use of colchicine. His most successful method was the aqueous drop technique. Seeds were germinated in petri dishes and as soon as the cotyledons separated, drops of 0.15 to 0.30 percent aqueous colchicine solution were placed on the growing points at three hour intervals. Duration of the experiments ranged from eight to 24 hours. The seedlings were washed in water immediately after the treatment was completed and allowed to recover in petri dishes three to five days before transplanting to light, well fertilized soil in pots.

Evans (11), obtained polyploids in red clover, white clover and alfalfa, by the application of a 2 percent aqueous solution of colchicine to seedling by the drop method. She noticed that in addition to the normal indicators of polyploidy such as larger stomata and pollen grains, that polyploid red clover appeared more pubescent. Examination under the microscope showed that there is no difference in the number of hairs, but the polyploid red clover has thicker and longer hairs than the diploid, making it appear more pubescent.

Burton (7), 1957, reported that aqueous colchicine seed treatments resulted in from 10 to 100 percent tetraploid sectors in Bahiagrass. Effective concentrations were, 0.4 and 0.8 percent for six hours, and 0.2, 0.4, and 0.8 percent for 48 hours.

There are a few reports in the literature where polyploidy has been obtained in vetch by the use of colchicine. Yamamoto (48) obtained V. sativa tetraploids with colchicine solution of 0.025 to 0.050 percent concentrations.

Hertzch (14) reported obtaining 4n to 10n polyploid V. villosa by the use of colchicine. Nordenskiold (25) reported improved self fertility in tetraploid V. sativa by crossing two different strains of tetraploid V. sativa.

Nitrous Oxide

Ostergren (27) reported success in doubling the chromosome number in Crepis capillaris by placing the plants in a gas chamber under ten atmospheres (147 pounds per square inch) of nitrous oxide for periods from four to six hours.

Later, Nygren (26), copying Ostergren's technique, was able to obtain many polyploids in the Melandrium species. He used various pressures of nitrous oxide for variable durations. Five atmospheres pressure for durations of four to seven hours produced the highest percent of polyploids. In some cases 100 percent of the seeds produced on treated plants were doubled.

PART I

HYBRIDIZATION IN VETCH

Materials and Methods

Twenty four different species of vetch were assembled from: the various Regional Plant Introduction Stations; Swedish Seed Association, Svalof, Sweden; the Botanical Garden, Nancy, France; the University of Perugia, Perugia, Italy; and the Agronomic Institute, Versailles, France.

Ten seeds of each species were germinated on moist filter paper in petri dishes and then transferred to four inch pots in the greenhouse.

As the seedlings grew, they were supported with thin bamboo stakes, four feet long. Because of the vigorous growth of some species, it was found necessary to stretch wires across the top of the greenhouse, about ten feet above the pots and suspend strings from the wires to the top of the stakes.

Temperatures in the greenhouse were maintained near 60°F and the seedlings were put under continuous illumination in effort to speed up growth and flowering. All species flowered under the continuous illumination, with the exception of V. cracca which bloomed sparingly in the summer under normal day length.

Many interspecific crosses were attempted between the 24 species grown, with special emphasis placed on crossing V. villosa, V. dasycarpa, V. sativa, V. pannonica, and V. atropurpurea. Also, numerous intraspecific crosses were made between entries of V. sativa.

In species with the raceme type infloresence, emasculation could be easily performed by pulling off the entire corolla tube, which at the same time removed the anthers which adhere to the inside of the tube. In species with single or multiple flowers sessile to the main stem this technique could not be used. It was necessary to remove the petals separately to expose the anthers which could then be removed with forceps.

Results

No interspecific hybrids were obtained between the five major agricultural species in which special emphasis was placed in this crossing program. However, several crosses were made between strains of V. sativa and the species V. cornigera (Fig. 8) and V. calcarata (Fig. 7).

From the initial planting, only a few crosses were made between these species. Therefore, in a second planting several plants of V. calcarata, V. cornigera and V. sativa were grown. Many crosses were then made between these species with special care to avoid any pollen contamination. Seed was readily set in these crosses, using either parent as the female.

There were three purple flowered species obtained in the seed assembled, V. atropurpurea, V. bengalensis, and V. onobrychoides. These species appeared quite similar morphologically and when crosses were made in all combinations, it was found that they intercrossed quite freely. On the basis of these observations it is suggested that V. bengalensis and V. onobrychoides are strains of V. atropurpurea.

Of special interest in this crossing program was the marked response in pod stimulation obtained in V. atropurpurea by the application of pollen from nearly every species to which it was crossed. Unfortunately, however, after ten to 15 days, the pods began to brown at the tips and eventually withered and dropped off.

Attempts to excise embryos from these pods before this browning began, were unsuccessful. Upon cutting away the ovary wall, the exposed ovules were noticed to have a slightly sunken spot in the center. When a thin slice was removed from the lateral surface of these ovules a hollow space was observed in the center, with no trace of a developing embryo.

A high degree of compatibility was found between strains of V. sativa in intraspecific crosses. Fifty-eight different hybrids were obtained from the 130 strains of V. sativa. Twenty-eight of these F_1 hybrids were planted in the East Lansing nursery and observed for disease and insect resistance, vigor and seed yield during the 1958 growing season. In several cases the F_1 hybrids out yielded either parent in seed production and showed more vigor.

Summary

Because of the high compatibility between V. calcarata and V. sativa, and their morphological similarity, it is suggested that a possible synonymy exists between these two species.

V. cornigera appears quite similar to V. sativa and the two species cross readily. Since in a survey of literature no reference could be found on V. cornigera, it is suggested that V. cornigera is a selection from V. sativa rather than a distinct species. Similarly, it is suggested that two purple flowered species, V. onobrychoides and V. bengalensis, which readily cross with V. atropurpurea are selections from V. atropurpurea.

Most strains of V. sativa, though highly self fertile, will cross readily with each other and in some cases hybrid vigor is exhibited.

Fig. 1. *Vicia villosa*, Hairy vetch
$2n = 14$

Vicia villosa, glabrescence, Smooth vetch
$2n = 14$

Fig. 2. *Vicia dasycarpa*, Woolypod vetch
$2n = 14$

Fig. 3. *Vicia sativa*, Common vetch
2n = 12 or 14

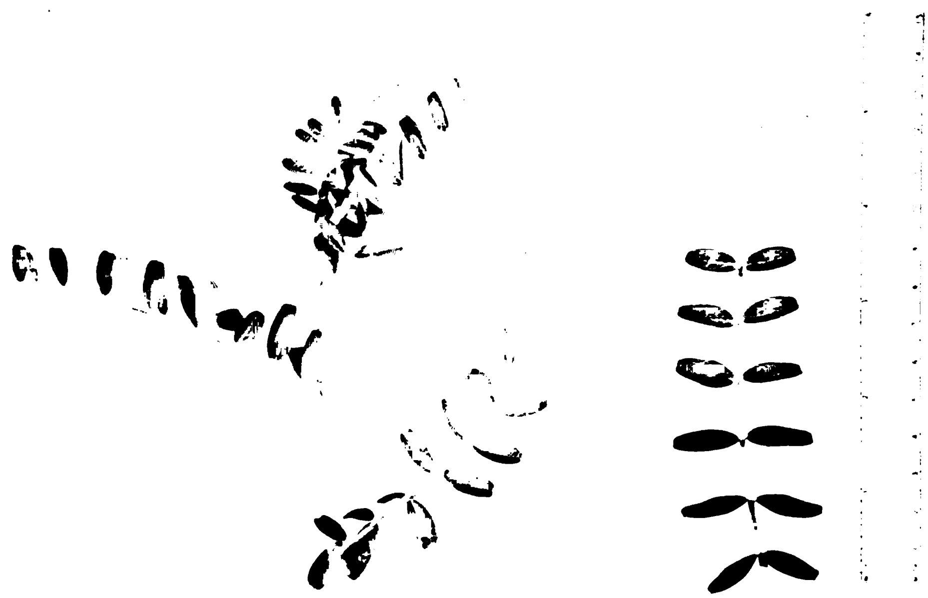
Fig. 4. *Vicia sativa*, *leucosperma*, Common vetch
2n= 12 or 14

Fig. 5. Vicia pannonica, Hungarian vetch
2n = 12

Fig. 6. Vicia atropurpurea, Purple vetch
2n = 14

Fig. 7. *Vicia calcarata*
$2n = 14$

Fig. 8. *Vicia cornigera*



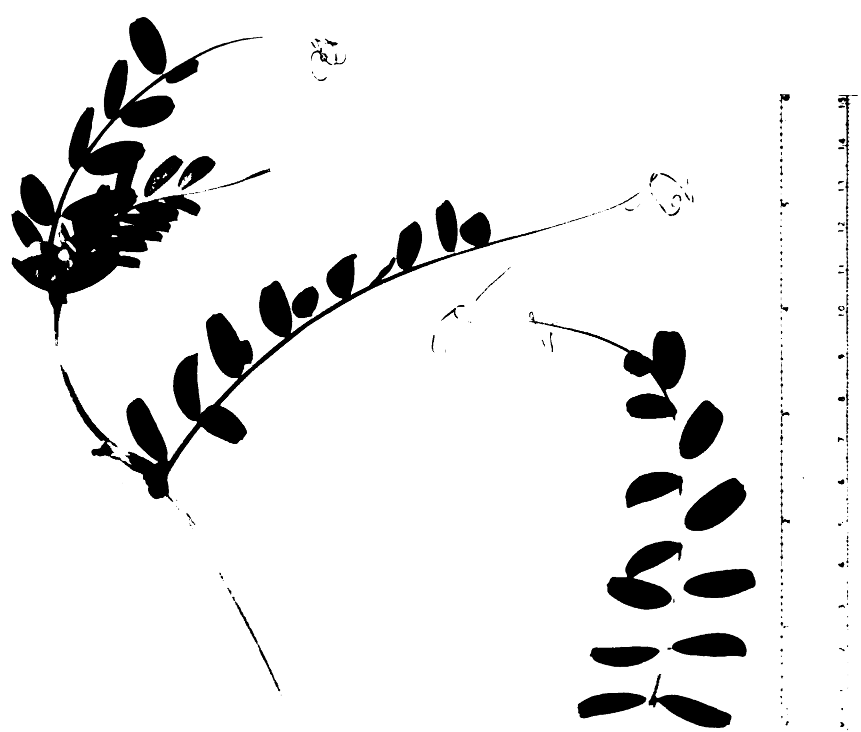


Fig. 9. *Vicia angustifolia*, Narrowleaf vetch
$2n = 12$

Fig. 10. *Vicia tetrasperma*, Sparrow vetch
$2n = 14$

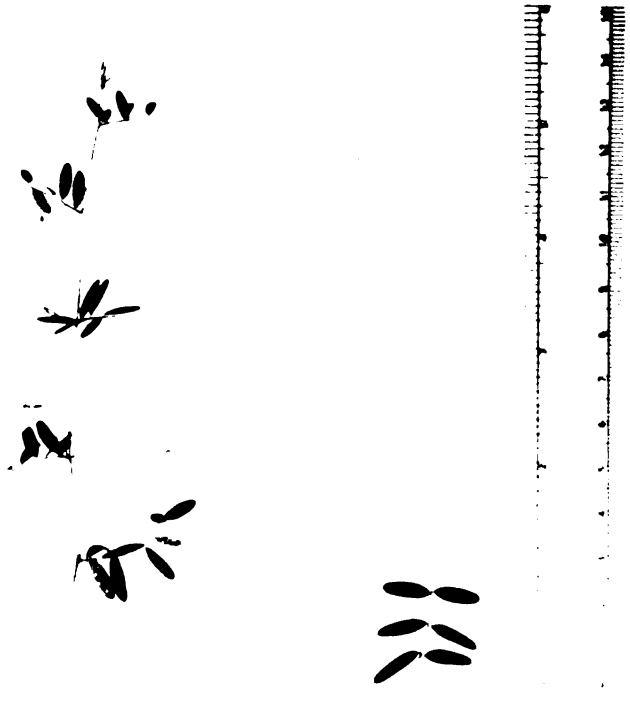


Fig. 11. Lens esculenta, Lentil
2n = 14

Fig. 12. Vicia cracca, Cow vetch
2n = 12, 14, or 28

PART II

PRELIMINARY EXPERIMENTS IN EMBRYO CULTURE

Materials and Methods

In general, the techniques developed by Keim (16) for culturing legume embryos were used, with modification where necessary. Four species of Vicia were used in these experiments, V. villosa, V. atropurpurea, V. sativa, and V. pannonica. The material was grown in the greenhouse at 60 ^{o} F in continuous light. Embryos were excised from ten to twenty-five days after pollination, varying in size from 0.5 mm in length to fully developed embryos.

A stereoscopic binocular was used with magnification of 20X. Care was taken to avoid contamination by cleaning the table top and spraying the room with 50 percent aqueous "ST 37" solution. Forceps and scalpel were used in the excision process. These were sterilized by flaming and placed in "ST 37" each time before use.

The entire pod was placed in the dissecting dish and while holding it with the forceps, the scapel was used to cut around the margin of the pod. Having done this, the pod was held firm with the scalpel and the top half of the pod peeled back with the forceps, exposing the ovules. The dissecting instruments were then again flamed and placed in "ST 37". Special care was taken to avoid tearing the ovule

loose from the ovary wall. It was much easier to hold the ovule firmly if it remained attached. Holding the ovule firmly with the forceps, the scalpel was used to cut a very thin slice through the lateral surface of the ovule wall exposing the embryo in a cavity of maternal tissue. The scalpel was then carefully eased beneath the embryo and it was lifted out. Then, holding the nutrient bottle slightly inclined downwards, the embryo was placed cotyledons down slightly into the agar medium. The instruments were then flamed and placed in "ST 37" until the procedure was repeated. It was found that a scalpel blunt on the end rather than pointed was more effective for removing the embryo from the ovule.

The glass containers used to hold the nutrient media were 2 dram vials, 15 by 75 mm, with cotton plugs. (Fig. 13) These proved to work satisfactorily until warmer weather caused excessive evaporation. Covering the top of the vials with aluminum foil aided somewhat in reducing evaporation losses.

The bottles containing the excised embryos were placed on a table top at room temperature under continuous florescent illumination. Attempts to grow embryos at constant temperature of 32° C and in the dark as suggested by Rappaport (32), were unsuccessful. At this higher temperature, the problem of evaporation was intensified.

An agar medium 0.7 percent, was used in all of these experiments. In general three levels (2, 4, and 8 percent) of sucrose were used in each experiment in an attempt to bracket the optimum sucrose concentration for embryos of varying stages of development.

The two dram vials were filled approximately half full by using the ring stand, funnel, rubber hose, and pinch clamp apparatus suggested by Keim (16). In this way, the vials could be filled rapidly without the media coming in contact with the upper portion of the vial. With the use of cotton plugs this was of particular importance as the plugs would stick in the vial, glued by the dried media. As the vials were filled, they were replaced in the original box which made it easy to handle and sterilize two to three hundred vials at one time. The media was sterilized for thirty minutes at 15 pounds pressure at 240° F. Since the vials were not pyrex, care was taken to raise and lower the temperature and pressure slowly to avoid breakage. Also, this avoided "bumping" of the media which might cause the media to come in contact with the cotton plugs or even pushing them out.

It was the intent of the writer to use embryo culture only as a tool to grow hybrid vetch embryos, not to do an intensive research in the field of embryo culture. Therefore, in general a simple media was used to begin with and as each failed to give the desired result, more elaborate media were tried. Since already established media were used

in these experiments, they will be referred to by the investigators who developed them, rather than listing the entire constituents of the media. Modifications to the media however will be specified.

Experimental Methods

The following preliminary experiments in embryo culturing were performed in effort to obtain a nutrient medium which could be used in the culturing of excised hybrid embryos in the Vicia species:

Experiment 1 -- Because of the success by Keim in growing clover and trefoil embryos on Randolph's medium, this was the first medium tried for the culturing of excised vetch embryos. Sucrose at the 2 percent level was used. (Table 1)

Experiment 2 -- Failure to grow embryos on Randolph's simple inorganic media plus sucrose suggested that a more complex medium might be needed. Rijven's basal medium with a complete source of trace elements was selected. To this medium were added the amino acids, glutamic acid and asparagine as the only nitrogen source. A stock nitrate solution was added to a portion of the media as a control. Sucrose was used at the 2 percent level. (Table 2)

Experiment 3 -- According to various investigators, the smaller the embryos at excision, the higher the sucrose concentration should be in the nutrient media. Therefore,

using the same medium as in experiment 2, the sucrose level was varied from 2 percent to 12 percent in hopes of obtaining optimum levels for embryos excised at various stages of development. (Table 3)

Experiment 4 -- Since a degree of success was obtained in the growing of nearly mature embryos in the asparagine supplemented media, variations in the concentration of asparagine and sucrose were used in an attempt to find a more optimum level for smaller embryos. (Table 4)

Experiment 5 -- Having had only partial success with Rijven's medium, a new media developed by Rappaport was tried. It was modified by adding Rijven's minor element solution and the vitamins, thiamine, niacin, and ascorbic acid. Three levels of sucrose were used. (Table 5)

Experiment 6 -- Because of the emphasis some investigators place on the level of sucrose, Randoph's media was used with sucrose at 1, 2, 3, and 4 percent sucrose. (Table 6)

Experiment 7 -- Since in previous experiments no success was obtained in growing small immature embryos, it was decided to try an organic medium. Van Overbeek's (41) coconut milk medium was tried, modifying it by using Rijven's basal medium and adding Van Overbeek's (41) extensive vitamin solution with the exception of adenine. (Table 7)

Experiment 8 -- Attempts to transfer the milk from the coconut aseptically to the nutrient meaium by use of a

hypodermic needle was found to be unsatisfactory. A high percent of contamination resulted. Therefore, it was decided to try the heat stable amino acid complex, casein hydrolysate which could be sterilized in the autoclave. The basal medium used was that of Rappaport's plus the addition of Rijven's trace element solution. The vitamin solution used by Sanders and Burkholder (36), in their casein hydrolysate media was also added. (Table 8)

Experiment 9 -- Upon purchase of a Seitz filter, the coconut milk media was again tried. The basal medium used was White's media, as modified by La Rue (19).

Asparagine was added to a portion of the basal medium and the sucrose concentration was varied. The coconut milk was used at nine parts coconut milk to one part media, as was found to be quite successful by Chang (8).

Experiment 10 -- La Rue's modified White's solution was again used, supplemented with asparagine and glutamine, (not glutamic acid as in previous experiments). In addition IAA was added as suggested by La Rue (19). (Table 10)

These embryo culturing experiments were began in December 1957. Ideal conditions were not available for a transfer room nor the growing of the embryos but an open room proved fairly satisfactory during the winter months. In the spring, however, as the temperatures increased in the culture room, rapid evaporation of water from the agar medium necessiated the termination of these experiments.

TABLE 1

Experiment 1

Randolph's Basal Medium	2% Sucrose

TABLE 2

Experiment 2

Rijven's Basal Medium Plus Sucrose, 2%	Glutamic Acid, 200 mg/l
	Asparagine, 200 mg/l
	Glutamic Acid and Asparagine each at 200 mg/l
	$Ca(NO_3)_2$, 75 mg/l KNO_3, 25 mg/l

TABLE 3

Experiment 3

Rijven's Basal Medium		Sucrose 2%
	Glutamic Acid, 200 mg/l	Sucrose 8%
		Sucrose 12%
	Asparagine, 200 mg/l	Sucrose 2%
		Sucrose 8%
		Sucrose 12%
	Glutamic Acid plus Asparagine each at 200 mg/l	Sucrose 2%
		Sucrose 8%
		Sucrose 12%
	$Ca(NO_3)_2$, 75 mg/l KNO_3, 25 mg/l	Sucrose 2%
		Sucrose 8%
		Sucrose 12%

TABLE 4

Experiment 4

Rijven's Basal Medium	Asparagine, 400 mg/l	Sucrose 2%
		Sucrose 4%
		Sucrose 8%
	Asparagine, 800 mg/l	Sucrose 2%
		Sucrose 4%
		Sucrose 8%
	Asparagine 1600 mg/l	Sucrose 2%
		Sucrose 4%
		Sucrose 8%
	Asparagine 2000 mg/l	Sucrose 2%
		Sucrose 4%
		Sucrose 8%

TABLE 5

Experiment 5

		Sucrose 2%
	*Thiamine HCl, 0.67mg/l Niacin, 0.67mg/l Ascorbic acid, 50.0 mg/l	Sucrose 4%
		Sucrose 8%
Rappaport's Basal Medium Rijven's Trace Element Solution		Sucrose 2%
	No Vitamin Solution	Sucrose 4%
		Sucrose 8%

TABLE 6

Experiment 6

	Sucrose 1%
	Sucrose 2%
Randolph's Basal Medium	Sucrose 3%
	Sucrose 4%

*Thiamine, niacin, and ascorbic acid have been shown to be beneficial in the growth of excised pea embryos by Bonner, et al. (2,3,4)

TABLE 7

Experiment 7

Rijven's Basal Medium	Coconut Milk 1 part to 500 parts medium	Van Overbeek's Vitamin Solution	Sucrose 2%
			Sucrose 4%
			Sucrose 8%
		No Vitamin Solution	Sucrose 2%
			Sucrose 4%
			Sucrose 8%
	No Coconut Milk	Van Overbeek's Vitamin Solution	Sucrose 2%
			Sucrose 4%
			Sucrose 8%
		No Vitamin Solution	Sucrose 2%
			Sucrose 4%
			Sucrose 8%

TABLE 8

Experiment 8

		Sucrose 2%
		Sucrose 8%
	**Casein, Cysteine, Tryptophane Solution, 100 ppm	Sucrose 12%
		Sucrose 2%
	Casein, Cysteine, Tryptophane Solution, 200 ppm	Sucrose 8%
		Sucrose 12%
Rappaport's Basal Medium Rijven's Trace Element Solution *Sanders and Burkholder's Vitamin Solution		Sucrose 2%
	Casein, Cysteine, Tryptophane Solution, 400 ppm	Sucrose 8%
		Sucrose 12%
		Sucrose 2%
	Casein, Cysteine, Tryptophane Solution 800 ppm	Sucrose 8%
		Sucrose 12%

* Thiamine HCl, 0.1 ppm; Niacin 0.8 ppm; Pyridoxine HCl 0.8 ppm
** This solution contains 6.67 mg each of cysteine and tryptophane per 100 mg of casein.

TABLE 9

Experiment 9

*La Rue's Modified White's Solution, Nitsch's Trace Elements	Asparagine, 400 mg/l	Coconut Milk, 9 parts to 1 part medium
		Sucrose 2%
		Sucrose 8%
		No Coconut Milk
		Sucrose 2%
		Sucrose 8%
	No Asparagine	Coconut Milk, 9 parts to 1 part medium
		Sucrose 2%
		Sucrose 8%
		No Coconut Milk
		Sucrose 2%
		Sucrose 8%

*White's medium was modified mainly by the addition of Nitsch's trace element solution plus 25 mg/l of $CoCl_2$, and a 2.5X increase in the vitamin concentrations plus 0.25 mg/l of Calcium Pantothenate.

TABLE 10

Experiment 10

*La Rue's Modified White's Solution, Nitsch's Trace Element Solution		IAA, 1 mg/l	Sucrose 2%
			Sucrose 8%
	Asparagine, 2000 mg/l		
			Sucrose 2%
		No IAA	
			Sucrose 8%
			Sucrose 2%
		IAA, 1 mg/l	
			Sucrose 8%
	Glutamine, 400 mg/l		
			Sucrose 2%
		No IAA	
			Sucrose 8%
			Sucrose 2%
		IAA, 1mg/l	
			Sucrose 8%
	Asparagine, 2000 mg/l Glutamine, 400 mg/l		
			Sucrose 2%
		No IAA	
			Sucrose 8%

*See Table 9.

Results

Of the 500 plus vetch embryos excised and cultured in these preliminary experiments, only six embryos, which were nearly mature at excision, showed indication of growth. These six embryos germinated seven to 12 days after excision and subsequently developed into normal seedlings. No indication of pregerminal growth was observed in either small or large embryos and no abnormal cell proliferation appeared.

1. One V. villosa embryo germinated seven days after culturing on Rijven's basal medium supplemented with 200 mg/l of asparagine and 2 percent sucrose (Table 2).

2. One V. atropurpurea embryo germinated ten days after culturing on Rijven's basal medium supplemented with 200 mg/l of asparagine, 200 mg/l of glutamic acid, and 2 percent sucrose (Table 2).

3. One V. villosa embryo germinated eight days after culturing on Rijven's basal medium supplemented with 200 mg/l of asparagine and 12 percent sucrose (Table 3).

4. One V. atropurpurea embryo germinated 12 days after culturing on Rijven's basal medium supplemented with 200 mg/l of asparagine, 200 mg/l of glutamic acid, and 12 percent sucrose (Table 3).

5. One V. villosa embryo germinated seven days after culturing on Rijven's basal medium supplemented with 800 mg/l of asparagine and 2 percent sucrose (Table 4).

6. One V. villosa embryo germinated ten days after culturing on Rijven's basal medium supplemented with 1600 mg/l of asparagine and 2 percent sucrose. (Table 4)

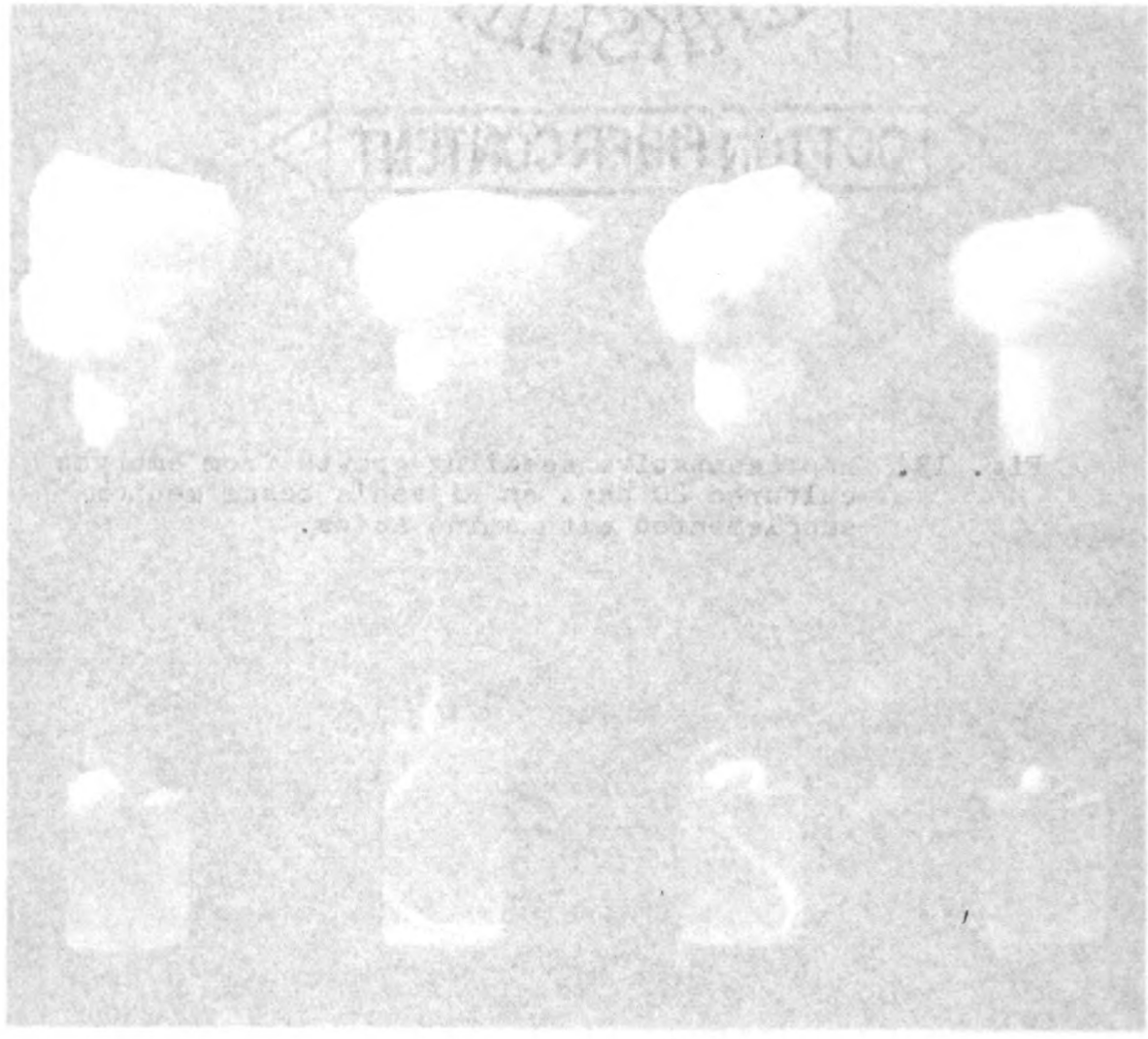
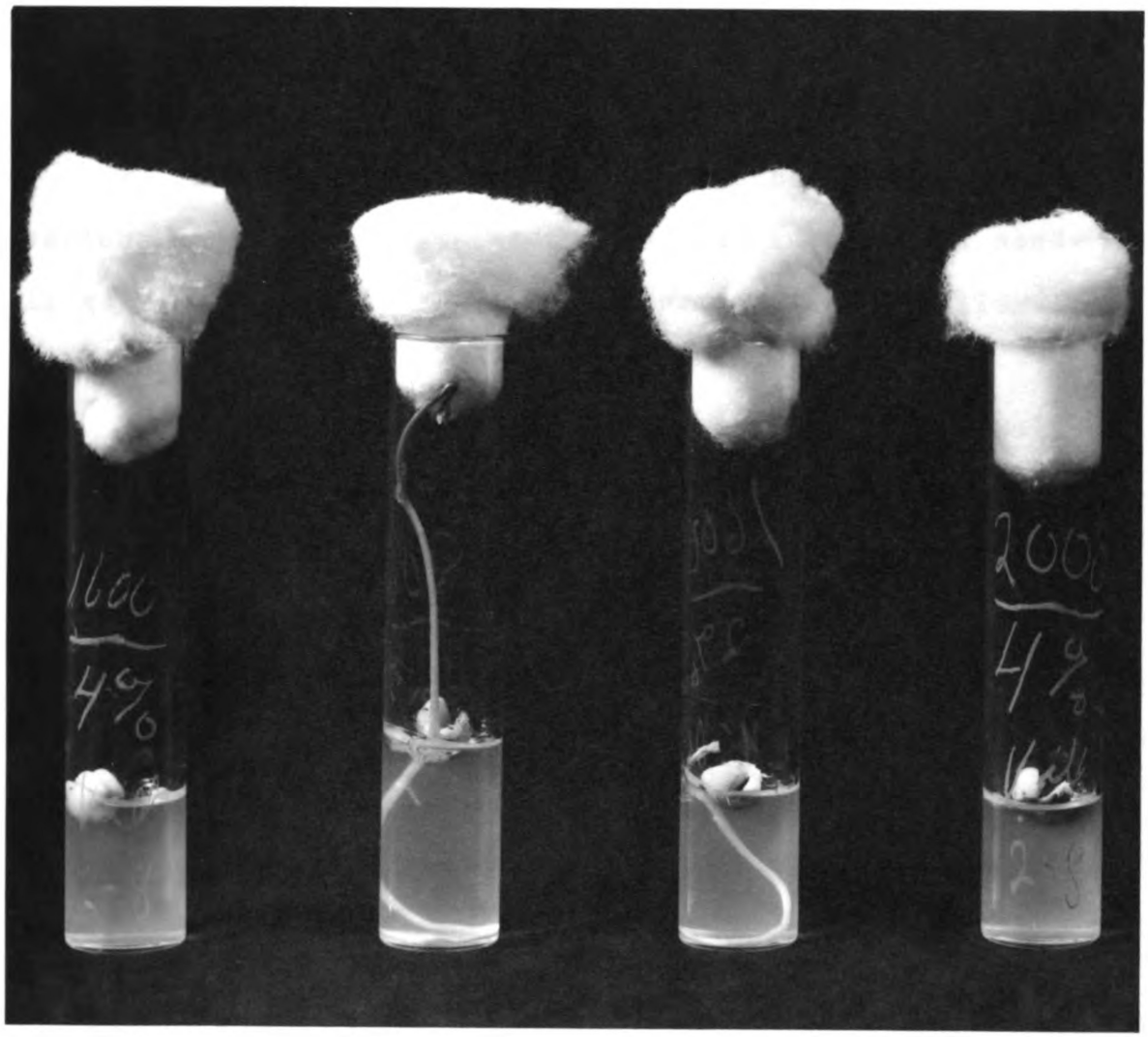


Fig. 13. Representative seedling growth from embryos cultured 20 days on Rijven's basal medium supplemented with amino acids.


1600
4%
2000
4%

SUMMARY

1. Preliminary experiments were run in effort to find a nutrient medium satisfactory for the culture of immature vetch embryos.

2. Six nearly mature embryos, excised and cultured in various media, germinated and developed into normal seedlings. No apparent growth was observed in any smaller immature embryos.

3. Each of the six embryos developing into normal seedlings were grown in a nutrient media consisting of Rijven's basal medium supplemented with sucrose and asparagine at various concentrations.

4. There appeared to be a species differentiation in nutrient requirements in that V. atropurpurea embryos required in addition to asparagine, a glutamic acid supplement. No V. villosa embryos grew in media supplemented with glutamic acid.

5. Concentration of asparagine and sucrose did not seem to be critical for embryos at the nearly mature stage of development.

6. No apparent benefit was observed from the addition of vitamins, IAA, and the organic supplements, coconut milk and casein hydrolysate.

7. Though emphasis was placed on the culturing of immature vetch embryos, this was not accomplished. However it is hoped that the information presented here will help give direction to further work in the embryo culturing of the Vicia species.

PART III

POLYPLOIDY INDUCTION

Because of failure to obtain many of the desired interspecies crosses at the diploid level attempts were made to develop a technique for obtaining polyploids in vetch, with the hope that species barriers could be broken down at a higher level of ploidy.

Two techniques were employed to obtain polyploidy. The first technique involved colchicine solutions in seed treatments and the second involved nitrous oxide gas under pressure. Each technique will be discussed separately.

Colchicine Treatments

Materials and Methods

This experiment actually consists of two sub-experiments. In the first experiment, four species of vetch were used, V. atropurpurea, V. villosa, V. sativa and V. pannonica. Colchicine solutions varying in concentrations from 0.2, 0.4, to 0.8 percent were prepared. The seeds were divided into two lots of ten seeds each for each species. One lot in each species was placed directly into the various colchicine solutions for intervals of ten to 24 hours, whereas the other half was first soaked 48 hours in distilled water and then transferred to the colchicine for six and 24 hours.

In the second experiment, only V. atropurpurea and V. villosa were used. Since it has been suggested that colchicine is most effective when cells are rapidly dividing, it seemed feasible that possibly the stimulating effect of gibberellin on germination and growth could be utilized to advantage. The same colchicine concentrations were used as in the previous experiment with the addition of gibberellin at 250 and 500 ppm. According to Wittwer and Bukovac(45), peas soaked over night in petri dishes with 250 ppm of gibberellin showed hastened germination and rapid emergence.

One lot of seed for each species was soaked over night in gibberellin and then treated with colchicine six and 24 hours. The other lot was placed directly into a mixture of gibberellin and colchicine for intervals of ten to 24 hours.

In both experiments, as each treatment was completed, the seeds were planted in rows in flats of sand in the greenhouse.

Results

The soaking of seeds in colchicine following germination in water was a more severe treatment than when the dry seeds were put directly into the colchicine. Many plants were stunted particularly at the higher concentrations of colchicine and 24 hours of exposure. Plants suspected of polyploidy on the basis of stunted and abnormal growth were transplanted from the flats to four inch pots of sterile soil. Of the 15 plants transplanted, only six survived. There

apparently was considerable root injury in these seedlings and they died before a root system became established. Of the six surviving plants, only one showed apparent tetraploid sectors based on larger flowers. This plant arose from a treatment where the dry seed was placed directly into 0.8 percent aqueous colchicine solution and soaked for 24 hours. Another characteristic later observed on this plant and which proved to be quite accurate for detecting tetraploid sectors from diploid sectors was a more noticeable pubesence on the leaves and stems. Examination under the binoculars showed that this increased pubesence was not due to more hairs, but rather longer and thicker hairs. This same characteristic was discovered in tetraploid red clover by Evans (11). Final identification was made by cytolgical examination of pollen mother cells which showed the doubled chromosome number of 28.

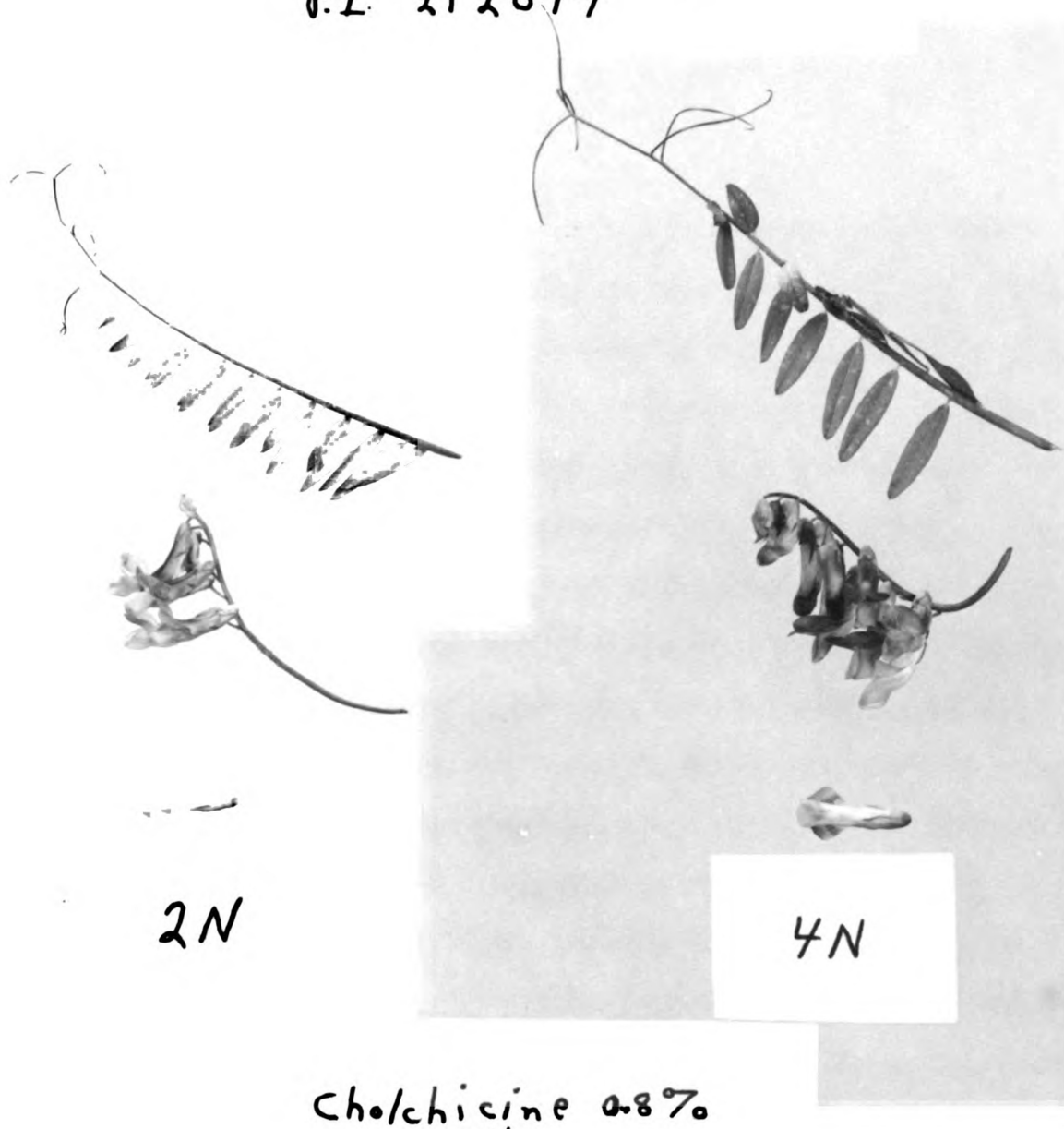
In the treatments to which gibberellin was added, a slight speed up in germination and growth of the seedlings was observed, but there was no indication that it enhanced the effect of colchicine.

Summary

Since several seeds germinated and produced normal seedlings after treatment in 0.8 percent colchicine solution for 24 hours, and the only polyploid plant produced was from this same treatment, it is suggested that higher concentrations of colchicine might be used more effectively.

Fig. 14. Comparison of diploid and tetraploid sectors produced on a colchicine treated V. villosa plant.

V. villosa, ardagon
P.I. 212044



Cholchicine 0.8%
24hrs

Root injury observed in plants from treated seed indicates that possibly seedling treatment with colchicine, in which the roots do not come in contact with the colchicine might be more successful.

Nitrous Oxide Treatments

Materials and Methods

Since it appeared that the colchicine treatments were not too effective, it was decided to try the technique developed and used by Ostergren and later used by Nygren, wherein they used nitrous oxide to obtain polyploids. Use was made of a gas chamber designed and set up by Dr. Elliott (10) for chromosome doubling work with other crops. (Fig. 15)

A major advantage of nitrous oxide doubling over colchicine is that the zygote can be treated at the first mitotic division, producing a completely doubled embryo with no chimeras. Often, doubled sectors of colchicine treated plants are crowded out by the more vigorous diploid sectors.

For nitrous oxide to be most effective, the zygote should be treated at its first mitotic division. Thus it was necessary to select a vetch species in which pollination could be easily controlled. Fortunately, in previous experiments it was found that V. atropurpurea could be easily emasculated and would readily set seed following hand pollination. Hence, this species was used in these experiments.

In order to bracket the right length of time from pollination to the first zygotic division, the plants were treated at 10, 13½, 17, 20½, and 24 hours after pollination.

Three different pressures were used, 50 pounds, 75 pounds, and 100 pounds. The duration of the treatments ranged from four hours up to 16 hours.

Some 130 plants of V. atropurpurea were grown to assure plentiful material. At flowering, a pollination schedual was set up so that one plant was pollinated every three and one half hours over a seventeen and one half hour period. The plants were then placed under treatment ten hours after the last pollination. In this way five plants covering the range desired for "hours from pollination," could be treated at one time. Usually five to ten florets were pollinated per plant.

In order to treat five plants at once, it was necessary to remove the plants from the pots. This was done carefully so as to disturb the roots as little as possible. It was found that if the pots were watered prior to removal of the plants, the soil held togetner better and also the moisture was beneficial to the plants during the treatment. The plants still staked up with four foot stakes were placed in a large plastic bag, long enough so the plants could be easily lowered into the gas chamber and easily pulled out when the treatments were completed.

After completion of a treatment, the pressure was slowly decreased, over a period of ten to 15 minutes, by gradually draining out the nitrous oxide. Before opening the gas chamber, it was first flushed out with nitrogen. More than one hundred plants were treated by this method, some of the treatments being repeated twice.

In preparation for mitotic chromosome counting of treated seedlings preliminary experiments were run with seminal root tips of V. atropurpurea seedlings. Root tips were first treated in oxyquinoline (20), then transferred to Newcomer's fixing solution (23), and finally stained with the propionic carmine smear technique (38).

The best stage for excision was found to be when the roots had emerged from five to ten mm beyond the seed coat. Earlier excision failed to show any actively dividing cells. Also, the time of day that excision was made, seemed to be critical, with from 11:00 A.M. to 12: A.M. consistently being the best time. The length of time left in the oxyquinoline seemed to be somewhat critical with about five or six hours being the optimum time. However some very shrunken cnromosomes were observed in material left in oxyquinoline for 24 hours. Root tips were left in Newcomer's solution for at least 48 hours before removal for staining.

Fig. 15. Gas chamber set up used for treating plants with nitrous oxide. Designed by Dr. F. C. Elliott (10).

Fig. 16. Somatic chromosomes of root tips treated at 6° C for six hours in 0.002 M oxyquinoline, 48 hours in Newcomer's solution and then fixed in propionic carmine. V. atropurpurea
2n = 14 1500 X


Nitrogen
NOX
NITROUS OXIDE

Results

As shown in Table 11, no seed was produced when the treatment was more severe than 75 pounds for 10 hours. Plants, however, did survive treatments of 100 pounds for 10 hours. When the duration of the treatment exceeded 16 hours, no seed was produced under any level of pressure and at 13 hours, the only seed produced was on plants under 50 pounds pressure.

Time did not permit chromosome determination of all seed, but a sample of at least two seeds per treatment was germinated and their root tips excised for chromosome counting. Using the preparation and staining technique developed in preliminary experiments, approximately 75 percent of the root tips examined were at the right stage of division for chromosome counting. From this sampling of seed, no polyploid cells were observed.

Summary

The application of nitrous oxide treatments are relatively easy and many seeds are produced in some treatments, each seed being a potential polyploid. Thus it can readily be seen from these experiments, that if any effective treatment were found, numerous polyploids of vetch could be produced without difficulty. In a sampling of seed from each treatment, no polyploids were found. However, it is hoped that from the 111 seeds produced on the treated plants, some polyploid seedlings will be obtained.

Table 11

Nitrous Oxide Treatments

Pounds Pressure	Hours	Hours after Pollination	No. of Plants	No. of Seeds
50	4	10	2	5
		13½	2	8
		17	2	7
		20½	2	18
		24	2	21
50	7	10	1	0
		13½	1	0
		17	1	0
		20½	1	2
		24	1	8
50	10	10	1	0
		13½	1	0
		17	1	0
		20½	1	0
		24	1	0
50	13	10	1	1
		13½	1	0
		17	1	0
		20½	1	0
		24	1	0
50	16	10	1	0
		13½	1	0
		17	1	0
		20½	1	0
		24	1*	0
75	4	10	2	0
		13½	2	5
		17	2	10
		20½	2	5
		24	2	4
75	7	10	1,1*	0
		13½	1,1*	0
		17	1,1*	0
		20½	1,1*	0
		24	1,1*	0

Pounds Pressure	Hours	Hours after Pollination	No. of Plants	No. of Seeds
75	10	10	2	12
		13½	2	4
		17	2	0
		20½	2	1
		24	2	0
75	13	10	1	0
		13½	1	0
		17	1	0
		20½	1*	0
		24	1*	0
75	16	10	1	0
		13½	1	0
		17	1	0
		20½	1	0
		24	1	0
100	4	10	2	0
		13½	2	0
		17	2	0
		20½	2	0
		24	2	0
100	7	10	2	0
		13½	2	0
		17	2	0
		20½	2	0
		24	2	0
100	10	10	1	0
		13½	1	0
		17	1	0
		20½	1	0
		24	1	0
100	13	10	1*	0
		13½	1*	0
		17	1*	0
		20½	1*	0
		24	1*	0
100	16	10	1*	0
		13½	1*	0
		17	1*	0
		20½	1*	0
		24	1*	0

* Plant killed.

GENERAL SUMMARY AND CONCLUSIONS

A strong incompatibility exists between species of Vicia. From numerous interspecific crosses made between 24 different species, the following crosses were successful;

V. sativa	X	V. calcarata
V. sativa	X	V. cornigera
V. atropurpurea	X	V. onobrychoides
V. atropurpurea	X	V. bengalensis
V. onobrychoides	X	V. bengalensis

These crosses set seed readily, with reciprocal crosses being equally fertile. However, because of the high degree of compatibility and morphological similarity between species, it is suggested that synonymy may exist between V. sativa, V. calcarata and V. cornigera, and similarly between V. atropurpurea, V. onobrychoides and V. bengalensis.

Intraspecific crosses within V. sativa exhibited high compatibility. Fifty-eight different hybrids were obtained from 130 entries of V. sativa. The F_1 hybrids, together with both parents were planted in the East Lansing nursery in 1958. In several cases the F_1 exhibited greater vigor and set more seed than either parent.

In V. atropurpurea, a strong pod stimulation was observed from interspecific crosses. Attempts to excise embryos from the poorly developed seeds in these pods were unsuccessful.

Failure to obtain hybrid embryos indicates that the barrier to interspecific hybridization is effective at fertilization or shortly after in these particular crosses.

Attempts to develop a nutrient medium for the culture of vetch embryos was only partially successful. Nearly mature V. villosa and V. atropurpurea embryos were successfully cultured on Rijven's basal medium supplemented with the amino acids asparagine and or glutamic acid. It is hoped that this observed stimulation in growth of vetch embryos by asparagine and glutamic acid may help lead to the development of a nutrient medium satisfactory for the growth of small vetch embryos. Such a nutrient medium would be a valuable tool in effort to overcome interspecific barriers in vetch.

Seed treatment of vetch with colchicine produced polyploids only at the most severe treatment, 0.8 percent colchicine for 24 hours. This indicates that seed treatment with higher concentrations of colchicine for longer durations may be more effective in producing polyploids.

Many seeds were produced from the nitrous oxide treatments. However, in a sampling from each treatment, no doubled seed was observed. If a critical treatment is found that will produce polyploidy in vetch, it seems likely that numerous polyploid vetches could be readily produced by this treatment.

It is hoped that these initial steps, in the development of a nutrient medium and polyploid induction techniques for vetch, may be of value to other investigators for further development and eventual use to overcome species incompatibilities in Vicia.

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