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# True seed production in garlic

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**Abstract** Despite a long history of obligate vegetative propagation, selected garlic clones can produce sexual seeds. By removing vegetative topsets from the inflorescence and cutting inflorescences from the underground bulb, 63 germinable seeds were produced from 11 garlic clones in Wisconsin. Protein analysis of the seedlings confirms their snygamic origin. The generation of new recombinants through sexual reproduction could have a major impact on garlic production worldwide.

**Key words** *Allium sativum* L. · Sexual reproduction Seed

## Introduction

Garlic (*Allium sativum* L.) is an important crop that has been cultivated since ancient times for both its medicinal and culinary benefits. It has been touted as an effective remedy for a myriad of ailments, ranging from tuberculosis to hair loss. More recently, garlic has received attention from the medical community for its possible roles in reducing the incidence of atherosclerosis and coronary artery disease, its anticoagulant and hypoglycemic properties, and its anticarcinogenic effects (Lau 1989; Fenwick and Hanley 1985b).

Today global production of garlic exceeds 2662000 metric tons, grown on 421000 ha. In the United States, it has an annual cash value of around $30 million (FAO Yearbook 1987; Fenwick and Hanley 1985a).

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Despite its worldwide economic value, classical breeding of the crop has remained impossible due to its obligate apomictic nature. Garlic reproduces almost exclusively by means of underground cloves or vegetative topsets in the inflorescence. The cause of this sterility has been variously attributed to several possible mechanisms: garlic could be a sterile hybrid resulting from the cross of two fertile ancestral species (Takenaka 1931, cited by Etoh 1985); garlic flower buds may be unable to compete with rapidly growing vegetative topsets in the inflorescence (Koul and Gohil 1970); the tapetum may degenerate before pollen mitosis (Novak 1972); a series of "degenerative-like diseases" such as rickettsia-like organisms may interfere with sexual reproduction (Konvicka 1973; Konvicka et al. 1978); or observed floral morphological abnormalities may contribute to sterility (Etoh and Ogura 1977). Since garlic has been propagated asexually for many generations, an accumulation of chromosome aberrations such as aneuploidy and translocations and/or inversions could also significantly reduce the incidence of balanced gametes.

Although garlic has generally remained unamenable to conventional plant improvement techniques, there has been recent success in the production of germinable garlic seed (Kononkov 1953; Etoh 1983, 1985; Etoh et al. 1988; Konvicka 1984). Establishing sexual reproduction in garlic has the practical significance of providing access to the genetic variation generated by meiosis and fertilization to enable breeding of the crop. Propagation through seeds instead of cloves could also yield economic benefits. It has been estimated that a virus-free crop yields 20% more than a virus-infected crop (Havranek 1974), and all clones for production must be disinfected of nematodes (*Ditylenchus dipsaci*). Seed propagation of garlic would be a more economical method to eliminate virus and nematodes than the labor-intensive meristem culture used today. Because seeds are smaller and easier to handle, store, and transport than cloves, it is also possible that production costs could be lowered substantially by a seed-grown crop. In addition to these economic benefits of garlic seed pro-

duction, the establishment of sexual reproduction would provide a means for evaluating the extent of variation in the garlic genome, the genetic factors which influence sexual reproduction, and the relationship between *A. sativum* and its undomesticated progenitor species *A. longicuspis*. The purpose of this study was to obtain true garlic seed from clones of a Northern American garlic collection.

## Materials and methods

### Plant material, flowers and seed production

For this study, 150–200 *A. sativum* and *A. longicuspis* clones from the USDA collection (Pullman, Wash.), Brazil, Germany, Poland, and the former Soviet Union were used. Ten to 20 cloves of each clone were planted between September 15 and October 15 outdoors in cold frames in Kegonsa silt-loam soil in 1987–1992 in Madison, Wis. Garlic plants flowered in mid-June to mid-July. Spathes of all flowering clones were opened manually when vegetative topsets or bulbils, which together with the flowers comprise the umbel, filled out the spathe completely, usually around the time of tetrad formation in microsporogenesis. After opening the spathe, vegetative topsets were removed with fine forceps. Topsets were not removed from approximately 10% of the umbels. Approximately 90% of the flower stems from umbels with topsets removed were cut 10–15 cm above the ground and placed in jars of water, with the remaining flowers left attached to the bulb in the ground. To reduce the deleterious effects of intense sunlight, heat or humidity, cut flower stems were placed under shade cloth outdoors (1989), or in air conditioned greenhouses at 22–26°C (1989, 1991–1992) or 16°C (1990). Some inflorescences were open-pollinated by insect vectors and others were hand pollinated using pollen from pollen-shedding clones. Garlic seeds were harvested in the early fall when the inflorescence dried and were stored at 3°C. Seeds were surface sterilized in 70% ethanol for 1 min, 10% commercial bleach for 1 min, and rinsed in sterile distilled water. Seeds were gently scarified with a scalpel and were placed on sterile solid B-5 medium (Gamborg et al. 1976) without hormones. Seedlings were transplanted to soil at the one-to-two leaf seedling stage and grown in the greenhouse until spring, at which time they were hardened off and planted outdoors.

### Pollen and cytological investigations

Fresh and preserved anthers were used for cytological observation of meiosis in microspore mother cells. Flowers at various stages of development (1.5–2.5 mm in length) were collected and stored in 70% ethanol until analysis. Anthers were squashed in 0.5% acetocarmine, heated, and observed through a light microscope at 400-fold magnification.

Pollen germination tests were conducted using fresh pollen. Drops of a mixture consisting of 15% sucrose and 1% agar were placed side-by-side on a microscope slide. Freshly harvested anthers were gently touched to the agar drop to release pollen and slides were placed in a covered petri dish containing moistened filter paper to maintain high humidity. After 24–36 h, a drop of Lacmoid-Martius yellow stain (Phillips 1981) was placed on each sample, followed by a coverslip. Germinating pollen tubes stained blue within 15 min of staining.

### Isozymes

Isozymes of seedlings were analyzed to confirm their sexual origin. Approximately 100 mg fresh leaf tissue from each seedling was ground with silica sand in 500 µl cold extraction buffer consisting of 0.05 M Tris pH 6.8, 0.2% dithiothreitol (DTT), 1% polyvinylpyrrolidone-40 (PVP-40), 10% dimethylsulfoxide (DMSO), and 0.05% β-mercaptoethanol. After 5 min refrigerated microcentrifugation, 5 µl glycerol/dye (0.5% bromphenol blue in 50% glycerol) was added to 100 µl supernatant. The resulting sample was frozen at −80°C until analysis.

Sample proteins were separated using 0.75-mm slab discontinuous polyacrylamide running gel (pH 6.8) and 4.5% stacking gel (pH 8.8). Samples were stacked (30 µl sample/lane) at 12.5 mA per gel and run for 2.5–3 h at 25 mA per gel. Gels were stained for esterase (100 mg diazo blue and 50 mg α-napthyl acetate dissolved in 20 ml 95% ethanol, then added to 100 ml 0.08 M Tris pH7.0), or for total protein using Coomassie stain (stain: 2.5 g Brilliant Blue R dissolved in 90 ml glacial acetic acid, 450 ml 100% methanol, 450 ml distilled $H_2O$; destain: 180 ml glacial acetic acid, 600 ml 100% methanol, 1220 ml distilled $H_2O$).

### Ovule culture

Ovule culture was attempted to facilitate embryo or ovule development. Green swollen ovaries were harvested from cut flower heads 2 to 20 days after pollination. Dried outer petals were removed and the whole ovary was surface sterilized in 70% ethanol for 1 min, 10% commercial bleach for 5 min, then rinsed in sterile distilled water. Whole ovaries or individual ovules were cultured 12 per petri dish on B-5 medium (Gamborg et al. 1976) with and without 1 ppm picloram or 10 ppm benzyladenine. Cultures were grown in the dark at 23°C.

## Results and discussion

### Flowers and seed production

Between the years 1988 and 1992, 63 germinable garlic seeds from 11 different garlic clones were produced (Table 1). To date, five of these seeds matured into bulb-forming plants in two years (Fig. 1). Based on the results of 5 years of seed production, it was apparent that environmental conditions contribute significantly to seed set. Our observations suggest that if flowers are exposed

**Table 1** Garlic seed production in Wisconsin 1988–1992

| Year | Approximate number of inflorescences | Approximate number of shrivelled seeds | Number of germinating seeds | Seed-producing garlic clones |
|---|---|---|---|---|
| 1988 | 150 | 10 | 2 | K/Ro, R54 |
| 1989 | 300 | 100 | 11 | R54, R83, DGru |
| 1990 | 500 | 25 | 0 | – |
| 1991 | 500 | 200 | 1 | DGru |
| 1992 | 500 | 320 | 49 | DGru, D6038, D6811, D6864 D7087, D7099, U073, U094 |

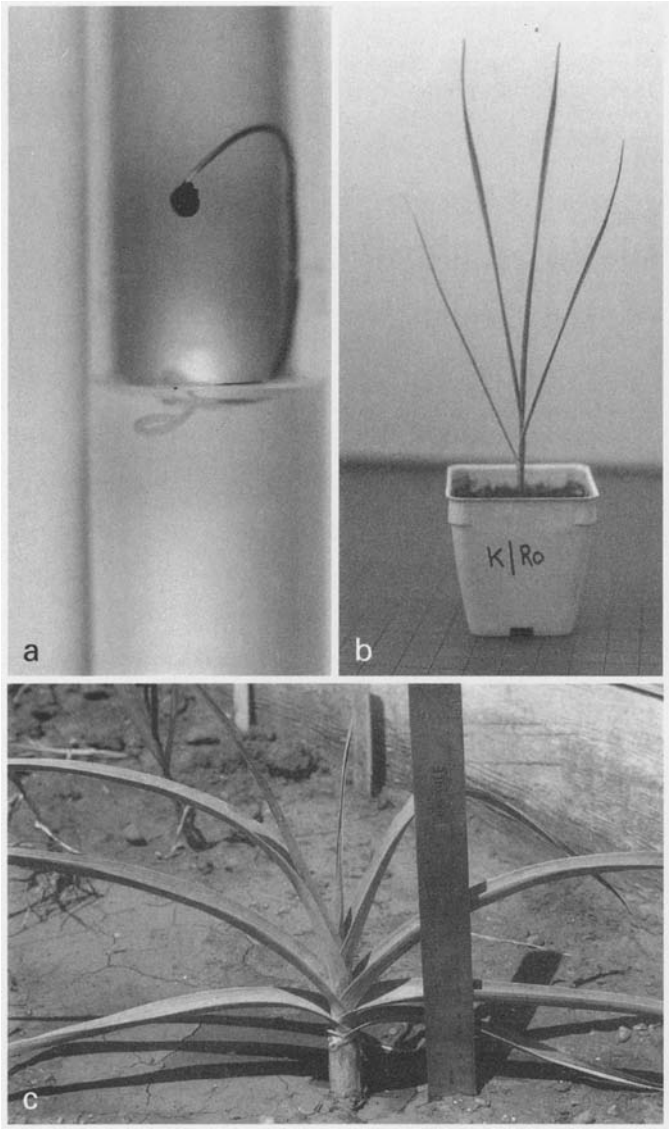


**Fig. 1** Growth of 1988 K/Ro garlic seedling: **a** 6-week-old seedling in sterile, hormone-free B-5 medium; **b** 5-month-old seedling; **c** 9-month-old seedling

to intense sunlight and heat (1988) or to prolonged temperatures below 65°F (18.3°C; 1990), seed set was greatly reduced. The success of Etoh et al. (1988) in obtaining over 3000 germinable seeds from 16 Asian clones confirms our hypothesis that garlic seed production is highly clone-specific and environment-dependent. Topset removal was critical for seed production success. If left on the inflorescence, vegetative topsets continued to grow and flowers dried before anthesis. In contrast to the result of Etoh et al. (1988), when topsets were removed but inflorescences were not cut from the underground bulb, flowers senesced soon after anthesis and no seed was set, whereas removal of the inflorescence from the bulb resulted in delayed senescence of floral parts. For both treatments, failure to yield garlic seed may have resulted from the successful competition of topsets or bulbs for

**Table 2** Fertility of USDA garlic collection at Wisconsin

| | 1991 | 1992 |
|---|---|---|
| Total number of clones | 210 | 206 |
| Number of flowering clones | 132 | 137 |
| Number of clones with non-senescing flowers | 43 | 61 |
| Number of clones with purple, pollen-shedding anthers | 26 | 27 |

**Table 3** Garlic pollen germination of selected clones (1991)

| Clone | Percent of pollen with pollen tubes | Percent of pollen grains stainable |
|---|---|---|
| M/N | 0 | 0 |
| R54 | 1.5 | 18.5 |
| R81 | 0.8 | 3.8 |
| R83 | 10.5 | 13.7 |
| RAL27 | 0.8 | 7.3 |
| RAL28 | 0.3 | 3.5 |
| RAL751 | 0.3 | 0.1 |
| U085 | 0 | 0 |

nutrients or from translocation of inhibiting compounds to the flowers.

Mature garlic seeds were similar to onion seeds but generally smaller and usually somewhat shrivelled. Empty seed coats were not uncommon and account for 80–90% of total seed set. Germination of seeds occurred in 2–3 days or required scarification, depending on the genotype and the vigor of the embryo. Etoh et al. (1988) have indicated that germination rate and seedling vigor vary with genotype and may be increased by cold, moist stratification. Although some seedlings were quite vigorous, approximately half of them suffered developmental abnormalities and died after reaching the 2–3 leaf stage. This lack of seedling vigor may have been due to mitotic abnormalities (untested), recessive deleterious loci, or inbreeding depression in self-pollinated seedlings.

## Pollen and cytological investigations

In the Wisconsin garlic collection, approximately half of the clones flowered and 10% of the clones shed pollen (Table 2). Like Konvicka (1984), we have found a consistent relationship between anther color and pollen shed: purple anthers shed pollen while yellow anthers do not. In the majority of garlic clones in our collection, microspores degenerate at or before the tetrad stage, or pollen grains are empty and do not stain with acetocarmine. Germination of pollen of selected clones was low, ranging from none to 10.5% (Table 3).

Cytological analysis of garlic clones revealed regular bivalent formation during microsporogenesis in all 29 clones examined (Fig. 2). This observation is in contrast

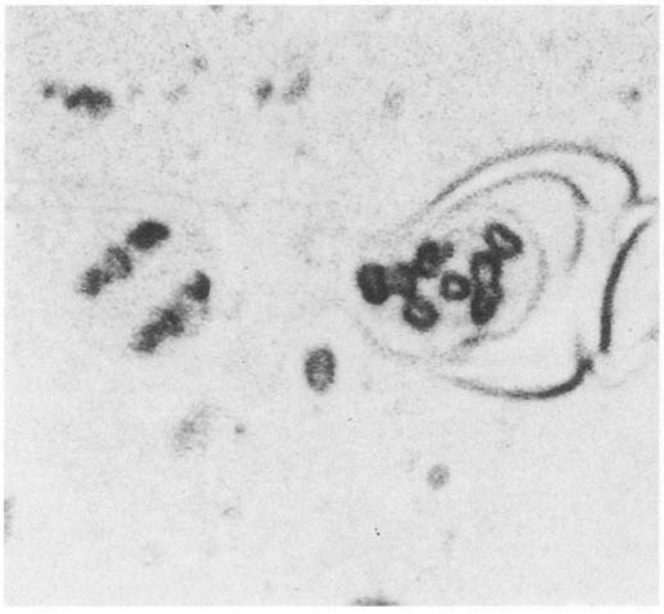

**Fig. 2** Regular microsporogenesis of garlic clone K/Ro at anaphase I (*left*) and diakinesis (*right*). Eight bivalents can be distinguished

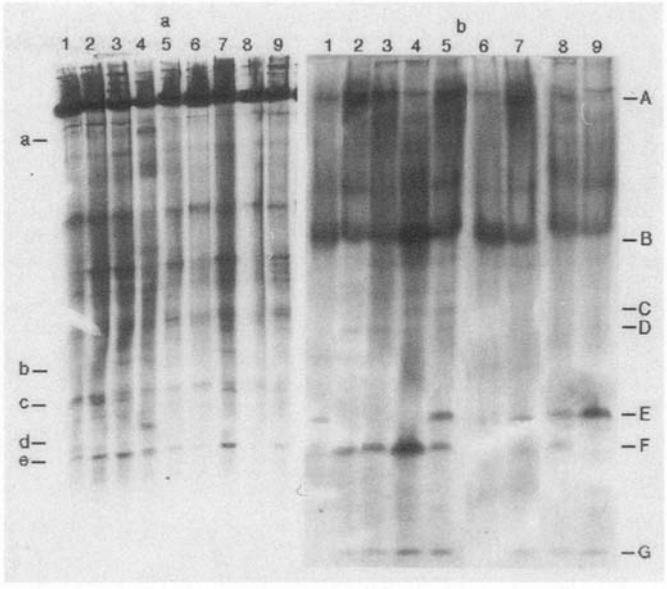


**Fig. 3a, b** Polyacrylamide gels of seedlings and their maternal parents. **a** Gel stained for esterase; **b** gel stained for total protein (Coomassie). *Lane* 1 Parent clone DDRGru, *lanes* 2–5 seedling progeny of DDRGru, *lane* 6, parent clone R54, *Lane* 7 seedling progeny of R54, *Lane* 8 parent clone R83, *Lane* 9 seedling progeny of R83. Bands which differ between parents and their progeny are labelled **a–e** for total protein, **A–G** for esterase

to the work of Etoh (1985), who observed a large number of meiotic abnormalities, especially multivalent formation. The difference could reflect a different genetic base, as most of the clones observed in this study were obtained from European or US collections, whereas most of Etoh's clones were from Asian collections.

## Isozymes

Total protein bands "a" to "e" and esterase bands "A" to "G" were present in seedlings but not their maternal parents (Fig. 3). This suggests that all the seed-derived progeny resulted from a sexual process, rather than an agamospermic event, such as parthenogenesis. Because parent clones DDRGru, D6038, D6811, D6864, D7087, R54, R83, U073, and U094 were pollen-fertile and were open pollinated, self-pollination may also be possible. Parent clone K/Ro and D7099 are pollen-sterile, and each produced one vigorous open pollinated seedling in 1988 (Fig. 1).

## Ovule culture

Rescued ovules developed black seed-like structures but did not result in viable embryos. It is not clear if this lack of development of embryos was due to cultural conditions or simply to a lack of successful fertilization.

In conclusion, the establishment of sexual reproduction in garlic could have a major impact on garlic production. Although seed-produced garlic may have economically significant ramifications in its virus-free nature and reduced planting costs, the real value in true seed garlic lies in the ability to improve garlic through conventional breeding techniques. Garlic exhibits an extensive amount of morphological and biochemical variation which, until now, has been locked up in a long history of asexual reproduction. The 200-clone garlic collection in Wisconsin demonstrates variation in economic traits such as clove size, skin color, flavor, number of cloves per bulb, and maturity date, and breeding-related traits such as flowering ability, anther color, pollen viability, and meiotic behavior (Pooler and Simon 1993). Variation between and within garlic clones has also been observed in disease resistance (Rengwalska and Simon 1986). Establishing sexual reproduction in garlic provides access to this variation by generating a large number of new recombinants, some of which will undoubtedly be valuable to the garlic industry worldwide.

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