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An improved method to obtain novel mutants in *Cucurbita pepo* by pollen viability

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ABSTRACT

A new genetic resource for *Cucurbita pepo* has been developed with chemically induced mutagenesis. The seeds of the zucchini cultivar MU-CU16 were treated with 40 mM–80 mM ethyl methanesulfonate (EMS), reaching high germination rates between 70 and 85%. However, most plants of those M1 populations did not produce offspring, and the fertility rates were lower in plants treated with higher concentrations of EMS. Once we established that visual flower abnormality rates were not sufficient to explain low fruit yield, pollen viability was analysed with fluorochromatic reaction. Compared with untreated plants, treatment with EMS produced a substantial decrease in pollen viability, and only the group of plants with pollen viability rates higher than 45% yielded nearly 70% of fruits with seeds. Therefore, the main issues to be addressed for developing mutant lines in this species are to increase the number of mutations in the genome and to increase the number of mutant lines with sufficient fertility. In this case, the early plantlet selection for high pollen viability carried out as part of this work represents a useful tool for use in future breeding programs by mutagenesis, allowing an increase of up to 40% in the production of mutant lines for a dosage of 65 mM EMS.

1. Introduction

Plant populations derived from mutagen-treated germplasm are potential sources of novel genetic variation. Screening and selecting for mutants within these populations have added some valuable new traits to crop gene pools, including short stature and high-yield rice in China, semi-dwarf durum wheat in Italy and tomatoes adapted to drought conditions in Cuba (Ahloowalia et al., 2004).

There are diverse mutagenesis strategies to generate new genetic variation, but chemical mutagens have gained popularity because they are easy to use, do not require any specialised equipment and can provide a very high mutation frequency. Amongst chemical mutagens, ethyl methanesulfonate (EMS) is currently the most widely used. Compared with radiation, which causes damage on a large scale and severely reduces viability (Sikora et al., 2011), chemical mutagens tend to cause single base-pair (bp) changes rather than deletions and translocations. EMS, in particular, provides a random-point mutation wherein G–C base-pairs (bps) are switched to A–T pairs (Hoffmann, 1980). This chemical-induced strategy has been used to generate new alleles for nodule development in *Lotus japonicus*, spike morphology in barley, lignin and β-glucan production in *Avena sativa* and extended shelf life in *Cucumis melo* (Kurowska et al., 2011).

However, serious difficulties have emerged in the attempt to produce mutant populations for some crop species, such as *Cucurbita pepo* L. This species is perhaps the most polymorphic with respect to fruit characteristics of the *Cucurbitaceae* (Duchesne, 1786; Naudin, 1856), comprising eight edible-fruited morphotypes (Paris, 1986). The zucchini morphotype is the most recently developed and has the least variability (Paris et al., 2003), but importantly, it is the most widely consumed by humans. The breeder's initial selection of traits for this morphotype included bush habit, increasing femaleness and early flowering that increased production and resulted in the first commercial hybrids. A low number of resistance genes was introgressed in commercial cultivars from genotypes of related species, such as *C. moschata* Duchesne, and wild relatives, such as *C. okeechobeensis* (small) Bailey. These species represent an existing source of genetic variation to incorporate required traits, but their use is limited by

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crossability barriers, including reduced fertility and quasi-linkage (Formisano et al., 2010).

Advantageous breeding results may be achieved by efficient selection methods on mutant populations, which require that the population size (number of families) compensates for the density of mutations (Parry et al., 2009). As zucchini plants are relatively large, the number of plants that can be grown per unit area is limited. Consequently, there is a need for an efficient screening procedure of small, young plants. Moreover, self-pollination is necessary to allow expression of recessive traits, but in allogamous crops having large plants, such as zucchini, the process of self-pollination and selection requires considerable field space.

The development of mutant populations is limited by the reduction in plant fertility often caused by mutagens. Reduced fertility can be expressed as less fruit and production of fewer seeds per fruit, forcing an increase in the number of lines to be screened or the use of milder applications, such as lower concentrations of a chemical mutagen, to increase fertility, but at the cost of the need for growing much larger plant populations (Caldwell et al., 2004). Determining the main parameters that influence fertility in *C. pepo*, such as pollen viability analysis, might enable more efficient production of an adequately sized mutant population. Assessments of pollen viability, together with artificial pollinations, have proven useful in monitoring sterility problems (Dent-Acosta et al., 1995; Rodríguez-Riano and Dafni, 2000) and could, potentially, help overcome them.

The goal of the present research was to develop an optimal protocol to establish an EMS mutant population of *Cucurbita pepo* as a first step towards efficiently obtaining novel variation in this species. Selection for increased fruit and seed production based on selection for increased pollen viability is to be tested as the foundation of an effective method to generate novel and potentially useful mutations in zucchini.

2. Materials and methods

2.1. EMS mutagenesis

Experiments were performed using seeds from the *Cucurbita pepo* subsp. *pepo* L. Zucchini Group 'MU-CU16'. This accession belongs to the *Cucurbita* core collection of the Cucurbits Breeding Group from the Institute for the Conservation and Breeding of Agricultural Biodiversity (COMAV).

Seeds of 'MU-CU16' were treated with EMS. First, to balance the maximum mutation density with an acceptable plant survival rate, an EMS "kill curve" analysis was established on batches of 100 seeds treated with seven different EMS concentrations ranging from 0 mM to 240 mM. The seeds were imbibed in bottles containing EMS at different concentrations and placed on a rotary shaker overnight (16 h) at room temperature. To end the EMS treatment, the seeds were treated with 0.2 mM sodium thiosulfate for 10 min at room temperature and rinsed with dH_2O for 30 min with gentle shaking. Finally, the seeds were placed on trays over wet paper overnight prior to planting.

2.2. Mutant population development

Concentrations of EMS deemed suitable for application to *C. pepo* seeds were those for which the "kill curve" showed germination rates higher than 60%. Finally, three optional concentrations were applied to produce the mutant populations and evaluate the effects on offspring production. Because increasing the EMS concentration decreases the germination rate, the number of seeds to be treated was increased for the highest EMS dosage applied. Mutagen-treated plantlets were sown, the plants grew and controlled self-pollinations were attempted on each plant. Female flowers were protected before anthesis to prevent the transfer of pollen by insects, and each M1 plant was self-pollinated by hand early in the morning. At 60–80 days after self-pollination, M2 seeds were extracted.

The presence of visible mutations in the first mutant generation was evaluated to determine how mutations altered reproductive development. To analyse how fertility was prevented in the mutant population, the plants were classified into three groups: those producing seeds, those producing fruits without seeds and those that failed to produce fruits.

2.3. Pollen viability analysis

Male flowers in anthesis from each group of plants were collected early in the morning and kept in a humid chamber for no more than 2 h. Pollen viability was determined by the fluorochromatic reaction test (FCR test, Heslop-Harrison and Heslop-Harrison, 1970). Subsequently, pollen grains were immersed in a freshly prepared solution of fluorescein diacetate made up of a concentration of approximately 10^{-6} M in sucrose 0.5 M (Cuevas and Pinillos, 2008). A few minutes later, pollen grains were observed under a fluorescence microscope (Olympus BX40F4). Viability was expressed as a percentage of fluorescent pollen grains, with at least 200 pollen grains counted per sample.

Pollen viability analysis was carried out in 40 mM and 80 mM EMS mutant populations because they were the most distant concentrations of mutagen in the range selected. The fluorochromatic reaction (FCR) test was performed on 200 M1 plants randomly chosen from each population and compared with results from untreated control plants. Plants were grouped into three categories depending on pollen viability rates: low, when pollen viability was less than 45%; medium, when pollen viability was between 45 and 90%; and high, when pollen viability was higher than 90%. Seed production was determined by counting seeds whose shape and size were similar to seeds produced by the untreated 'MU-CU16', which had a germination rate of 100%.

2.4. Selection by pollen viability

An early selection of plants by pollen viability was performed in two groups of 200 M1 plants treated with 40 mM and 65 mM EMS. These concentrations were chosen to improve the fruit and seed recovery from mutant plants in the range of previously selected concentrations. The groups were then arranged into two randomised blocks. Each group was divided into two halves including plants selected or unselected by pollen viability rates.

Young selected M1 plants were self-pollinated and those having pollen viability, of the first male flower, of greater than 45% were compared with unselected plants. Fruits were allowed to mature and the seeds of each fruit were counted.

The results were subjected to analysis of variance in a 2-factorial design, one factor being with and without pollen selection and the other the dosage of EMS. Means of significantly different results ($p < 0.05$) were compared with Tukey's Test.

3. Results

3.1. Production of EMS Cucurbita pepo mutant population

Three concentrations of EMS, 80 mM, 65 mM and 40 mM, were chosen for large-scale mutagenesis, as they showed germination rates of approximately 70 to 85% (Fig. 1). Approximately 7000 seeds that were mutagenized with 80 mM EMS were sown. The resulting M1 plants were self pollinated but only 6% of the plants produced seeds (Table 1). Of the approximately 4000 seeds subjected to the 65 mM EMS treatment that were sown, only 9% of the M1 plants