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Biochemical evaluation of germplasm regeneration methods for cucumber, *Cucumis sativus* L.[1]

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Summary

During the 1980s, the North Central Regional Plant Introduction Station replaced open-pollinated samples of *Cucumis sativus* with samples regenerated by cage pollination. Enzyme polymorphisms, detected by horizontal starch gel electrophoresis, were evaluated to document genetic changes in 157 *Cucumis* accessions resulting from changes in seed-regeneration methods. Analysis of levels of sample homozygosity indicates that cage-pollinated samples are significantly more homozygous than the open-pollinated samples they replaced, but the two groups of samples differ little in overall allelic composition. The frequency of rare alleles also has not changed significantly, although three alleles were found in cage-pollinated samples that were not noted in their older counterparts. The results of this study may not be interpreted as conclusive support for the use of cage pollination for regeneration of *Cucumis* germplasm collections. But the comparison of within-accession levels of homozygosity suggests that the genetic integrity of individual accessions is maintained better with cage pollination than with less-controlled regeneration methods. Only a genetic comparison of original seed samples with regenerated samples can conclusively document the effectiveness of any regeneration system.

Introduction

Since 1949, the North Central Regional Plant Introduction Station (NCRPIS) has maintained germplasm collections of cucumber, *Cucumis sativus* L. Until 1978, seeds were regenerated under open-field conditions by controlled pollination. This system had several important drawbacks. The plants were exposed to insect-transmitted diseases, sib pollinations had a variable success rate, and the process was labour intensive. When sib pollinations failed, open-pollinated fruits had to be harvested.

The genetic integrity of these populations could be compromised by this regeneration system. Changes in gene frequencies could occur from unintentional selection for insect and disease resistance, from genetic drift resulting from small harvests in years when many sib pollinations failed, and from contamination resulting from the harvest of open-pollinated fruits. Levels of contamination from the use of open-pollinated fruits of *C. sativus* are unpredictable and can vary from insignificant to important, as shown by field experiments that examined gene flow patterns by using a dominant genetic marker (Handel, 1983).

These shortcomings led to the testing of a new system: regeneration by using honey bees (*Apis mellifera* L.) in screened cages. This system was tested in 1978 and adopted as a standard procedure of the NCRPIS in 1979. Ellis *et al.* (1981) described this system and its advantages for regeneration of *Cichorium, Daucus, Medicago, Melilotus* and *Petroselinum*, as well as for *Cucumis*.

Since 1979, the NCRPIS has been systematically replacing seed samples resulting from open pollination with samples resulting from bee pollination. The plants grown for this purpose have been produced either from remnant original seeds or from control-pollinated samples derived from original seeds.

In 1987, 757 *Cucumis* accessions from the NCRPIS collection were sampled to assess their genetic diversity (Knerr *et al.*, 1989). Ground cotyledon tissue was screened for genetic variation by horizontal starch gel electrophoresis using 40 enzyme systems. Knerr *et al.* (1989) identified 18 provisionary polymorphic enzyme-coding loci among the 757 accessions surveyed. Knerr and Staub (in press) determined that polymorphisms for 14 of these 18 loci were inherited in a simple Mendelian fashion and that a predictable genetic basis could not be ascribed to the other isozymes.

The accessions screened by Knerr *et al.* (1989) included 433 samples produced by caged pollination. The other 324 samples resulted from open pollinations, sib pollinations and pollinations with different degrees of physical isolation. Between 1987 and 1989, 157 of these 324 samples were discarded at the NCRPIS and replaced by seeds from cage isolation.

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The 157 replacement samples have now also been evaluated for genetic variation by horizontal starch gel electrophoresis. This presents an unusual opportunity to compare the overall genetic diversity and structure of a large number of germplasm collections produced by different regeneration methods. Three questions of interest to users and managers of *Cucumis sativus* germplasm collections can be examined.

1. Does the method of regeneration significantly change overall gene frequencies in these 157 accessions?
2. Does the occurrence of rare alleles in these accessions change with regeneration method? More specifically, are there rare alleles present in the assemblage of cage-pollinated samples that are not found in open-pollinated counterparts?
3. Because accessions may have a history of isolation and given that *C. sativus* is somewhat tolerant to inbreeding (Jenkins, 1942; Robinson and Whitaker, 1974), individual accessions may be more homozygous than expected from random mating. Are samples regenerated by cage pollination more homozygous than those from open pollination?

Although this study can help answer these questions, it is important to remember that studies based on overall gene frequencies cannot evaluate the genetic integrity of individual samples. Only comparison of gene frequencies of original seed samples with those of regenerated samples can conclusively document the effectiveness of any particular regeneration method.

Materials and Methods

In February 1990, samples of 157 *C. sativus* accessions from the NCRPIS germplasm collection were chosen for electrophoretic analysis. These particular samples were chosen to compare the genotypes of populations produced by cage increase with those from open pollination. All samples selected were produced by cage increase, and corresponding samples of these same 157 accessions had been evaluated previously (Knerr *et al.*, 1989). Of these samples, 138 resulted from open pollination, three had an open-pollinated increase in their pedigrees, and the other 16 were produced with unknown levels of physical isolation. Complete pedigrees were determined for 101 of the 138 samples resulting from open pollination. Four of the 101 pedigrees included two generations of open pollination, the others had only one open-pollinated generation.

In March 1990, cotyledons of 15 seedlings from each accession were harvested, bulked, and prepared for electrophoretic analysis following the procedures of Knerr *et al.* (1989). Electrophoretic techniques and isozyme nomenclature also follow those of Knerr *et al.* (1989). To standardize relative band mobilities, extracts of the *C. sativus* inbred line, GY-14a, were loaded on each gel, and band mobilities were recorded in relation to the bands of GY-14a.

Each accession was evaluated for variation at the following polymorphic loci: G2dh, Gpi-1, Gr-1, Idh, Mdh-1, Mdh-2, Mdh-3, Mpi-2, Pepla-2, Peppap-2, Per-4, Pgd-1, Pgd-2 and Pgm-1. The presence or absence of all known alleles at these 14 loci were then compared with similar data collected by Knerr *et al.* (1989) by using a two-sample, chi-square goodness-of-fit test (Gibbons, 1976) for each polymorphic enzyme system. The results of these tests were combined by using the procedure, 'Combined Probabilities from Independent Tests of Significance' of Sokal and Rohlf (1981), to make an overall comparison of the replacement samples with those originally evaluated by Knerr *et al.* (1989).

Rare alleles were defined as those present in less than 10% of the sum of the samples evaluated in this study and those evaluated by Knerr *et al.* (1989). Because the cotyledons from 15 seedlings were bulked for the analysis of each sample, it was not possible to calculate exact frequencies of rare alleles. Thus, estimated frequencies were calculated for rare alleles with the formula:

$$\hat{q}_i = \frac{6x_i + 3y_i + 2z_i}{942}, \text{ where}$$

$\hat{q}_i$ is the estimated frequency of allele i, x_i is the number of accessions that displayed only allele i, y_i is the number of accessions that displayed allele i and a second allele, z_i, is the number of accessions that displayed allele i and two other alleles, and the constant, 942, is six times the number of accessions evaluated in the experiment. Estimated frequencies for the two groups of samples were compared by using a two-sided, ordinary sign test (Gibbons, 1976).

For each accession, the number of homozygous enzyme systems was recorded and compared with data collected by Knerr *et al.* (1989) in a pairwise fashion with the one-sided, sign-test statistic for large samples, $z_{+,R}$ (Gibbons, 1976). This statistic was chosen to test the hypothesis that samples regenerated by cage pollination were more homozygous than those from open pollination.

Results and Discussion

Table 1 presents a comparison between the samples of *C. sativus* evaluated by Knerr *et al.* (1989) and cage-pollinated replacements for 14 enzyme loci. There is no statistically significant difference in overall enzyme composition for these two groups of samples (Table 1). Significant shifts in enzyme composition did not occur with changes in regeneration methods.

Although no overall difference was found, there could still be significant changes in the frequencies of rare alleles. Table 2 presents a comparison of estimated gene frequencies for rare alleles between samples evaluated by Knerr *et al.* (1989) and cage-pollinated replacements. Once again, no statistically significant difference between the two groups of samples was found. Rare alleles may not have changed substantially under different regeneration methods, but one should note that three alleles found in the

Table 1. Comparison of allozymes present in cucumber accessions evaluated by Knerr *et al.* (1989) and cage-pollinated replacement samples

Locus	Alleles present	Samples used by Knerr *et al.*	Cage-pollinated samples
G2dh	1 & 2	0	1
	2	157	156
Gpi-1	1 & 2	1	1
	2	156	156
Gr-1	2	157	157
Idh	2	157	157
Mdh-1	1	157	156
	1 & 2	0	1
Mdh-2	1 & 2	2	0
	2	155	157
Mdh-3	1	153	154
	1 & 2	2	2
	2	2	1
Mpi-2	1	31	31
	1 & 2	90	95
	2	32	28
	1 & 3	3	2
	2 & 3	0	1
	1, 2 & 3	1	0
Pepla-2	1 & 5	1	0
	2 & 5	2	1
	3	1	1
	3 & 4	1	1
	3 & 5	2	3
	4	4	11
	4 & 5	36	27
	5	110	113
Peppap-2	2	157	157
Per-4	1	156	152
	2	1	5
Pgd-1	1 & 2	0	1
	2	157	156
Pgd-2	1	19	21
	1 & 2	83	64
	2	55	72
Pgm-1	1	33	27
	1 & 2	88	77
	2	36	53

χ^2(22 df) = 26.65, $0.2 < p < 0.3$, calculated using the procedure, 'Combined Probabilities from Independent Tests of Significance' (Sokal and Rohlf, 1981) (see text).

Table 2. Comparison of estimated frequencies, $\hat{q}$, of rare alleles present in cucumber accessions evaluated by Knerr *et al.* (1989) and cage-pollinated replacement samples

Allele	$\hat{q}$ for samples used by Knerr *et al.*	$\hat{q}$ for cage-pollinated samples
G2dh(2)	.000	.003
Gpi-1(1)	.003	.003
Mdh-1(2)	.000	.003
Mdh-2(1)	.006	.000
Mdh-3(2)	.019	.013
Mpi-2(3)	.012	.010
Pepla-2(1)	.003	.000
Pepla-2(2)	.006	.003
Pepla-2(3)	.016	.019
Per-4(2)	.006	.032
Pgd-1(1)	.000	.003

No significant difference between these two groups of samples was detected with the two-sided, ordinary sign test (Gibbons, 1976).

cage-pollinated samples, G2dh(1), Mdh-1(2) and Pgd-1(1), were not found in the corresponding samples used by Knerr *et al.* (1989). The loss of these three alleles may be a function of insufficient sampling for isozyme analysis or may reflect a real change. Although the frequencies of rare alleles have changed little overall, certain alleles may have been lost completely because of open pollination.

The two groups of samples were compared in a pairwise fashion, to see whether a sample evaluated by Knerr *et al.* (1989) was more or less homozygous than its cage-pollinated counterpart. Fifty-nine accessions showed no difference in homozygosity between the samples. Samples evaluated by Knerr *et al.* (1989) were less homozygous for 65 accessions and were more homozygous for 33 accessions. If changes in genetic structure were randomly distributed, one would expect these two numbers to be approximately equal. But in this analysis, the samples used by Knerr *et al.* (1989) were about twice as likely to be less homozygous than to be more homozygous, with the one-sided, sign-test statistic for large samples, $z_{+,R}$, equal to 3.131, supporting the hypothesis of a decrease in homozygosity, across all loci, with open pollination at the 0.1% significance level.

Conclusion

Replacement of open-pollinated samples of *C. sativus* with those produced by using the cage-pollination method of Ellis *et al.* (1981) has taken place at the NCRPIS during the 1980s. We have evaluated enzyme polymorphisms by using electrophoresis to document genetic changes in the germplasm collection that have resulted from replacing the open-pollinated samples.

Analysis of levels of homozygosity indicates that cage-pollinated samples are significantly more homozygous than the open-pollinated samples that they replaced, but the two groups of samples differ little in overall allelic composition. The frequency of rare alleles also has not changed significantly, although three alleles were recorded in the cage-pollinated samples that were not noted in their older counterparts.

The results of this study may not be interpreted as conclusive support for the use of cage pollination for regeneration of *Cucumis sativus* germplasm collections. This may, in part, be due to the fact that in most cases only one generation of open pollination occurred and that the degree of contamination may have been variable (Handel, 1983). But the comparison of within-accession homozygosity, combined with an expectation that individual accessions should be more homozygous than expected from random mating, suggests

that the genetic integrity of individual accessions is maintained better with cage pollination than with less-controlled regeneration methods.

The effectiveness of cage pollination methods for regeneration of *C. sativus* germplasm collections remains to be properly tested. Only a comparison of gene frequencies of original samples with samples produced by controlled tests of cage pollination methods can demonstrate the level of integrity of these methods. Such factors as population size, unusual sex ratios in certain accessions, and the phenology of flowering and fruiting may interact with cage pollination methods and should also be investigated.

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Résumé

Evaluation biochimique des méthodes de régenération du matériel génétique pour le concombre, Cucumis sativus *L.*
Dans les années 80, la Station régionale d'introduction des plantes pour le centre-nord a remplacé les spécimens de *Cucumis sativus* à pollinisation libre par des spécimens régénérés par pollinisation en cage. Les polymorphismes des enzymes, détectés par électrophorèse horizontale en gel d'amidon, ont été analysés pour illustrer les modifications génétiques de 157 spécimens de *Cucumis*, résultant de l'emploi de méthodes différentes pour la régénération des semences. L'analyse des niveaux d'homozygotes montre que les spécimens à pollinisation en cage sont beaucoup plus souvent homozygotes que les spécimens à pollinisation libre qu'ils remplacent, mais les deux groupes de spécimens diffèrent peu quant à leur composition allélique globale. La fréquence des allèles rares ne change pas beaucoup non plus, bien que trois allèles qui ont été détectés dans les spécimens à pollinisation en cage n'aient pas été notés dans les spécimens précédents. Les résultats de cette étude ne soutiennent guère la thèse de l'utilisation de la pollinisation en cage pour la régénération des collections de matériel génétique de *Cucumis*. Cependant, la comparaison des niveaux d'homozygotes au sein des mêmes spécimens suggère que l'intégrité génétique des différents spécimens est mieux conservée par la pollinisation en cage que par des méthodes de régénération moins contrôlées. Seule une comparaison génétique des spécimens de semences originaux avec des spécimens régénérés permettrait de conclure à l'efficacité de telle ou telle méthode de régénération.

Resumen

Evaluación bioquímica de los métodos de regeneración de germoplasma del pepino, Cucumis sativus *L.*
En los años ochenta, la Estación Regional Centro-Septentrional de Introducción de Plantas sustituyó las muestras de *Cucumis sativus* regeneradas en polinización abierta por muestras regeneradas mediante polinización en cajas. Se evaluaron en 157 muestras de *Cucumis,* mediante electroforesis horizontal en gel de almidón, los polimorfismos enzimáticos detectados para documentar las variaciones genéticas derivadas de los cambios en los métodos de regeneración de las semillas. El análisis de los niveles de homozigosis de las muestras indica que las polinizadas en cajas son considerablemente más homozigóticas que las muestras de polinización abierta sustituidas, pero son escasas las diferencias entre los dos grupos de muestras en cuanto a la composición alélica general. Tampoco cambió significativamente la frecuencia de alelos raros, aunque se encontraron tres alelos en muestras polinizadas en caja no observados en las otras. Los resultados de este estudio no se pueden interpretar como apoyo definitivo al empleo de la polinización en cajas para la regeneración de las colecciones de germoplasma del *Cucumis*. Sin embargo, la comparación de los niveles de homozigosis en cada muestra parece indicar que la integridad genética de cada una de ellas se mantiene mejor con la polinización en caja que con los métodos de regeneración menos controlados. Para demostrar definitivamente la eficacia de uno de los sistemas de regeneración es necesaria una comparación genética de muestras de semillas originales con muestras regeneradas.