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Nodulation and nitrogen fixation mutants of pea, *Pisum sativum*

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Summary. The 36 mutants which did not nodulate and 24 mutants which formed inefficient nodules with no or very low acetylene reduction activity were isolated among 86,000 M_2-seedlings of 'Finale' pea, *Pisum sativum* L., after treatment with chemical mutagens. One mutant was found for approximately every 50 chlorophyll mutants. Most mutations were induced by ethyl methanesulfonate; some by diethyl sulfate, ethyl nitrosourea and acidified sodium azide. Putative mutants were selected as nitrogen deficient plants, yellowing from the bottom and up, when M_2 seedlings were grown in sand with a *Rhizobium* mixture and PK fertilizer. The mutants were verified in the M_3 generation by acetylene reduction assay on intact plants.

Key words: Mutants – Chemical mutagenesis – Pisum – Nodulation – Symbiotic nitrogen fixation

Introduction

Mutants of legumes unable to nodulate or fix nitrogen may have important uses in investigations of the physiology and molecular biology of symbiotic nitrogen fixation. Natural nod$^-$ or fix$^-$ variants have been described in clover (Nutman 1984), soybean (review Caldwell and Vest 1977), alfalfa (Peterson and Barnes 1981), pea (Lie 1984; Ohlendorf 1983) and *Vicia faba* (Duc et al. 1985). Induced mutations for nitrogen fixation have been studied in pea (Jacobsen 1984; Jacobsen et al. 1985; Kneen and LaRue 1984, 1985; Messager 1985), chickpea (Davis et al. 1985) and soybean (Gresshoff et al. 1985; Mathews et al. 1985). A few such mutants, particularly the nitrate-resistant nodulators (Carroll et al. 1985; Jacobsen and Feenstra 1984) may be useful in plant breeding; some mutants can be used as controls in ^{15}N field experiments, but most will have mainly scientific interest. The purpose of the present work was to establish a collection of nitrogen fixation mutants, useful in studies on the role of the host in pea symbiotic nitrogen fixation.

Material and methods

The low, determinate, white flowered pea, *Pisum sativum* L., 'Finale' (Cebeco, Rotterdam, The Netherlands) with round, green seeds was used because of its stable yields, wide adaptation and its cultivation in many European countries (Bond 1984). 'Finale' seeds are quite rugged and not damaged by soaking like many other pea seeds. The 1 kg or 1.5 kg lots of seeds were soaked in twice as much mutagen solution (Table 1).

The mutagen treatments followed the protocols in the IAEA Manual on Mutation Breeding (1977). After use, the ethyl methanesulfonate (EMS) and diethyl sulfate (DES) were decomposed with NH_3, ethyl nitrosourea (ENU) with sulfuric acid followed by neutralization, and the acidified azide (NaN_3) was neutralized.

The seeds were treated dry or after presoaking for 18 h in deionized water. After treatment, they were washed in many changes of deionized water for half an hour, treated with cap-

Table 1. Mutagen treatments with ethyl methanesulfonate (EMS), diethyl sulfate (DES), ethyl nitrosourea (ENU) and acidified sodium azide (NaN_3)

Dry seeds			Presoaked seeds, 18 h		
EMS	0.1%	16 h	EMS	0.5%	1 h
EMS	0.2%	16 h	EMS	0.5%	2 h
DES	0.2%	16 h	EMS	1.0%	1 h
ENU	0.025%	16 h	EMS	1.0%	2 h
ENU	0.05%	16 h			
NaN_3	2 mM	3 h	NaN_3	2 mM	2 h

tan fungicide and sown wet in the field at 60 plants m^{-2}. At maturity one pod was harvested per plant.

The harvested M_2 seeds were sown in the greenhouse in sand in 60×30×8 cm plastic trays, 1 row per pod, 10 pods per tray. The seeds were treated with a mixture of captan and propamocarp fungicides before they were covered. The trays were inoculated with a mixture of *Rhizobium* strains and fertilized with the nitrogen-free, modified Wilson and Reisenauer (1963) nutrient solution containing 1 mmol/l KH_2PO_4, 2 mmol/l K_2HPO_4, 3 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 0.02 mmol/l $FeSO_4$, 0.02 mmol/l Na_2EDTA, 0.005 mmol/l $MnCl_2$, 0.002 mmol/l $ZnSO_4$, 0.25 mmol/l H_3BO_3, 0.0005 mmol/l $CuSO_4$, 0.0005 mmol/l $NaMoO_4$.

The temperature was kept at 10 °C at night and about 18°–22 °C during the day. After germination, the number of plants were counted. The chlorophyll mutants were counted and removed. When the plants reached the 6–8 leaf stage, depending on the season, plants yellowing from the bottom and up, indicating nitrogen deficiency, were pulled up and examined for nodulation. Plants without nodules as well as plants with white, green or very small nodules were classified as putative mutants and replanted in K-soil, a commercial potting mixture. M_3 seeds were harvested, sown in a K-soil : sand mixture and examined in the pot for nodulation and nitrogen fixation as acetylene reduction at the 6–8 leaf stage.

The acetylene reduction was determined on intact M_3 plants taken out of their pots (LaRue and Kurz 1973) in 30×22×12 cm acrylic plastic boxes, sealed with electrician's tape. The plants were incubated with 2% acetylene for 1 h, and the ethylene measured on a Hewlett-Packard gas chromatograph at very high sensitivity. The results were compared with non-mutant controls. Plants evolving less than 20% ethylene compared to the controls were considered verified mutants. They were potted in larger pots and grown to maturity.

Results and discussion

The results show that the mutation treatments given in Table 1 go from slight to quite severe. The results of the treatments are given in Table 2. A treatment of 0.025% ethyl nitrosourea overnight gave a low mutation frequency, measured as per cent chlorophyll mutants, while a 2 h treatment with 1% ethyl methanesulfonate after presoaking gave the very high mutation rate of 38% chlorophyll mutants, but very few surviving M_2 plants. Many mutants were weak and segregated other mutants in later generations. The 0.05% ethyl nitrosourea gave a quite high number of mutants.

During the initial screenings of the M_2 plants, several hundred putative mutants were isolated. Many of these could not be confirmed in the acetylene reduction assays in the M_3. Some of the putative mutants were chlorophyll mutants with a slow development of symptoms, and some M_2 plants showed nitrogen deficiency symptoms because the nodules were damaged by infection, usually by *Pythium* sp.

Nodulation and nitrogen fixation mutants can be isolated in quite high numbers. They are not rare at all. In this study I found one nitrogen fixation mutant per approximately 1,500 M_2 plants or 50 chlorophyll mutants. These numbers are probably minimum figures because some mutants have probably escaped detection in the rather simple selection system used in this work. The figures are comparable to the mutation rates given by Kneen and LaRue (1985) and Messager (1985).

The high number of mutants obtained indicates that many host genes are involved in the nodulation and nitrogen fixation of peas. This is not unexpected, as nodule formation and nitrogen fixation consist of many different steps.

The screening methods used by different groups have varied considerably. Most have screened individual plants in water culture (Kneen and LaRue 1984; Jacobsen 1984) or in sand (Carroll et al. 1985) or Puozzolane (Messager 1985). A much quicker but less sensitive method involving nitrogen deficiency symptoms has been used by Davis et al. (1985) and myself, where only plants showing nitrogen deficiency symptoms were examined.

Table 2. Number of nitrogen fixation pea mutants obtained in the M_2 generation, all verified by acetylene reduction assays on intact 6–8 leaved plants in the M_3 generation

Treatment	Non-nodulating mutants	Non-fixing mutants	No. of M_2 plants	Chlorophyll mutants, % M_2	Fertile M_1 plants	Seed sown kg
EMS 0.1% 16 h	10	2	14,462	3.1	4,400	2.5
EMS 0.2% 16 h	3	2	2,258	7.7	1,246	1
Presoak, EMS 0.5% 1 h	2	0	11,223	1.5	3,060	1.5
Presoak, EMS 0.5% 2 h	2	3	9,534	1.7	2,468	1.5
Presoak, EMS 1% 1 h	4	2	5,835	7.0	2,179	1
Presoak, EMS 1% 2 h	1	1	797	38.1	670	1
NaN_3 2×10^{-3} M, 3 h	1	3	10,821	2.2	2,340	1
Presoak, NaN_3, 2×10^{-3} M 2 h	1	3	8,903	3.5	2,160	1
DES 0.2% 16 h	2	1	5,772	1.2	1,130	1
ENU 0.025% 16 h	2	0	7,697	0.8	1,510	1
ENU 0.05% 16 h	8	7	8,920	3.3	2,370	1
	36	24	86,222	3.2		

Some of the mutants obtained have variable phenotypes. A mutant may have white nodules in one environment and red nodules in another, although acetylene reduction may be low in both cases.

Most of the mutants reported will be available for distribution. A few of the mutants have been unstable and some have died or set so little seed that propagation was not worthwhile.

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