EXPANDING THE GENETIC VARIABILITY OF FLATPEA USING TISSUE CULTURE, MUTAGENESIS, AND INTERCROSSING TECHNIQUES

by

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(ABSTRACT)

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Flatpea (Lathyrus sylvestris L.) is a potentially valuable forage legume but contains high levels of 2,4-diaminobutyric acid (DABA), a compound that can have adverse effects on some animals, including rats and poultry. To increase genetic variability in foliar DABA content and other traits of interest, three approaches were utilized: (1) regeneration of flatpea plants from tissue culture to produce potential somaclonal variants, (2) seed irradiation and screening of potentially mutated progeny, and (3) intercrossing among flatpea accessions. Low-frequency whole plant regeneration of flatpea was obtained from hypocotyl-derived callus cultures. Auxin concentrations above 5.0 μM resulted primarily in root formation without shoots. Conditions for optimum whole plant regeneration were as follows: callus was initiated on a modified Schenk-Hildebrandt low-ammonium medium containing 5.0 μM IAA (indole acetic acid) and 2.5 μM zeatin for callus initiation; after two subcultures, calli were transferred to a regeneration medium containing 0.1 μM IAA and 1.0 μM zeatin. Calli and regenerated root tissue contained 47 and 38% as much DABA as leaf tissue from 'Lathco' flatpea plants, respectively.

Initial tests established that the effective range of gamma irradiation for seed treatment was between 10.0 and 17.5 kR. Within this range, reduction in percentage of both seedling height and plant survival was a linear function of dose. Individual M_2 plants that contained reduced levels of DABA were identified. No significant trend in DABA concentration with increasing gamma irradiation was apparent.

Flatpea pollination methods were evaluated prior to utilization of intercrossing for inducing genetic variability. Appropriate flower stages for emasculation were determined by in vitro germination of pollen. Lines that produced high numbers of seeds per pollination were identified by crossing in all possible combinations among seven flatpea accessions. Cross-pollinations resulted in significantly higher seed set than selfing. Pollination using caged honeybees was the most efficient method of hybridization. Greater range and coefficient of variation in DABA concentration were observed for flatpea accessions than for intercrossed progeny produced by honeybee pollination.

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This dissertation is dedicated to the memory of

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Chapter I

INTRODUCTION

Flatpea is a highly promising conservation and forage legume that possesses extreme longevity. Stands of flatpea have been located which are more than 50 years old. Flatpea roots spread extensively and have been reported to penetrate to depths of 10 to 12 meters, permitting vigorous growth even in nutrient-deficient, acidic or droughty soils. Due to the shade tolerance, climbing ability, and dense tangled growth of flatpea, it can exclude weedy vegetation, but is easily controlled with herbicides. When utilized as a forage crop, flatpea hay can yield 9.0 to 11.2 Mg ha^{-1} and contain 20 to 30% protein; residue remaining after threshing of ripe seed may have as much as 15% protein. Vigorous growth and retention of leaves until a heavy frost permit flatpea pastures to be utilized late into the growing season.

Flatpea also has certain disadvantages which must be taken into account when considering its establishment. Weed control is essential during establishment to avoid crowding of young plants. Also, an impervious seedcoat restricts seed imbibition and seeds may lie in the ground for many months before sprouting, resulting in an uneven stand. However, scarification and imbibition of seeds prior to planting accelerates establishment and production of uniform stands is possible in 1 or 2 years.

Forage palatability, digestibility, and protein content of flatpea are excellent, but certain nonprotein amino acids, primarily 2,4-diaminobutyric acid (DABA), have been isolated from flatpea and shown to induce neurotoxic symptoms in rats and chicks. Although cases of sheep and rabbit deaths following flatpea consumption have been reported, defined studies have not verified any neurotoxic symptoms in livestock consuming flatpea forage.

Most flatpea seed stocks in the United States are derived from an extremely narrow genetic base. The single flatpea cultivar available, 'Lathco', would be improved by selection for both reduced DABA and more

rapid establishment. Selection for flatpea traits within Lathco germplasm requires expansion of genetic variability. Intercrossing has traditionally been used to increase variability in cross-pollinated crops such as flatpea. Prior to utilization of intercrossing techniques, the mode of pollination and effective crossing methods in flatpea need to be determined. Additionally, mutation induction using gamma irradiation has been effective in inducing variability in neurotoxin content of *Lathyrus sativus* L., a relative of the flatpea. Mutagenesis also has potential for inducing morphological changes such as altered flower color that can be used as marker traits to verify cross-pollinations. Induction of somaclonal variation using tissue culture techniques also provides a potential means of generating flatpea variability as well as a tool for studying DABA biosynthesis and metabolism. The objective of this study was to widen the flatpea germplasm base using a combination of tissue culture, induced mutations, and intercrossing among accessions. Resulting seed stocks may be useful for continued screening and selection for improved flatpea genotypes.

Chapter II

LITERATURE REVIEW

BOTANY

Flatpea (Lathyrus sylvestris L.) is a perennial, herbaceous legume which is characterized by rhizomatous, woody rootstocks and winged stems that may grow 0.9 to 1.8 m or more in length. Leaves are composed of a single pair of lanceolate to elliptical leaflets with parallel veins. Winged petioles terminate in five to seven branched tendrils. Nectiferous flowers are pink-colored and are borne in loose racemes of 3 to 12 blossoms. Blooming takes place over an extended period from mid-June to mid-August and a single plant may carry flower buds and well-developed pods at the same time. Seeds are black, globose or oblong, with a leathery appearance. The hilum extends one-half of the seed circumference. Flatpea forms a dense, tangled mass 0.9 to 1.2 m deep when growing in a full stand. If provided with support, plants climb as high as 2.4 to 3.0 m (Grunder and Dickson, 1948; McWilliams, 1970; Senn, 1938).

Taxonomically, L. sylvestris is classified in the section Eulathyrus of the genus Lathyrus which is within the Vicieae tribe and the Papilionoideae sub-family of the Leguminoseae. Lathyrus species have a common gametic chromosome number ($n = 7$) and are predominantly diploid ($2n = 14$) (Senn, 1937). The species most closely related to L. sylvestris which fall in the Eulathyrus section of the genus include L. heterophyllus, L. grandiflorus, L. latifolius, L. odoratus, L. tingitanus, L. tuberosus, and L. rotundifolius (Ascherson and Graebner, 1906-1910).

HISTORY

Flatpea, a native of Europe and the Caucasus region of Asia, was introduced to the United States by approximately 1888 by William Wagner, an agricultural professor from Wurtemberg, Germany (Piper, 1916; Wagner, 1943). Between 1888 and 1900, many trial plantings were made by U.S. experiment stations and private individuals. Experiment station reports praised flatpea, citing its large forage yields, high protein content, and ready consumption by livestock (Smyth, 1892; Clute and Mumford, 1893; Piper, 1916). However, flatpea failed to gain popularity with American farmers due to unavailability and high cost of seed, difficulty in establishment, and conflicting reports on toxicity and palatability of the forage (Pellett, 1941; Piper, 1916).

Revival of interest in flatpea did not occur until after 1931 when seed was brought to the Western Washington Experiment Station from the remnants of an old stand at the Michigan Agricultural Experiment Station and placed in trial plantings (Grunder and Dickson, 1948). Hodgson and Knott (1936) reported that sheep readily ate flatpea forage with no apparent ill effects. Sun-cured flatpea hay cut at full bloom was found to contain 25.3% crude protein with an average digestibility coefficient of 78.3%. Yields of 9.0 to 11.2 Mg ha^{-1} of flatpea hay were obtained by Daniel and Ensminger (1945). They also reported that cattle and sheep consumed flatpea forage with no harmful effects and noted the suitability of the crop for cut over timber lands in western Washington. Maximum forage yields of 11.2 Mg ha^{-1} with two cuttings of flatpea at early bloom were reported by Grunder and Dickson (1948). Reduced yields were found when two to four cuttings were made at earlier growth stages. Crude protein content dropped steadily from 40.78% at the 15.2-cm immature growth stage to 21.66% at maturity. Early and full-bloom crude protein contents were 30.85 and 27.11%, respectively. Daily gains of cattle grazing flatpea on logged-off pasture were very high early in the season but dropped off rapidly as the season advanced (Grunder and Dickson, 1948). The authors concluded that flatpea requires careful

management and will not survive under continuous close cutting or grazing.

NEUROTOXIN CONTENT

Beginning in the mid-1940's, efforts were begun to assess the potential toxicity of flatpea seed and forage to experimental animals. Researchers were likely motivated by the relatively recent outbreaks of lathyrism in India and the Mediterranean area. In humans, this disease is characterized by muscular weakness and paralysis of the lower legs, primarily in adolescent males. Lathyrism has been associated with ingestion of unusually large amounts of *Lathyrus sativus*, *L. cicera* and *L. cylmenum* peas during times of famine (Liener, 1967).

Adverse effects from consumption of flatpea have been documented in feeding trials utilizing poultry and rats. Miller et al. (1948) fed chicks *ad libitum* for 6 wk on diets in which the protein supplementation was partly or wholly ground defatted flatpea forage which had been harvested at pre-bud stage. Chicks gained only 95 g over 6 wk on 100% flatpea with 40% mortality compared to an average 551 g weight gain with no mortality for 100% soybean meal. Surviving chicks grew rapidly after the 100% flatpea diet was changed to a herring meal ration. In an earlier study, Miller (1946) had found that the continued feeding of seed at a 50% level resulted in death to 15-day-old chicks. Young rats fed a diet of 50% *L. sylvestris* pea meal developed neurological symptoms resembling those of human lathyrism (Lewis et al., 1948). The toxic substance(s) were extractable in 30% alcohol and extracted meal gave satisfactory growth. Similar results were obtained for *L. sylvestris* pea meal fed to young white mice (Lewis and Schulert, 1949). Huang et al. (1950) fed rats of varying size ground flatpea seed with or without autoclaving and supplementation of various B vitamins. All rats eventually died in every treatment, leading these workers to conclude that the toxic factor is thermostable and not an anti-vitamin.

Ressler et al. (1961) reported that a neuroactive principle identified as 2,4-diaminobutyric acid (DABA) had been isolated from seeds of

L. latifolius and L. sylvestris. During purification, fractions were bioassayed by administering aqueous extracts via stomach tubes to weanling rats. Rats developed weakness in the hind legs, tremors in upper-extremities, followed by convulsions and death. Chicks fed a diet containing 1.0% DABA grew poorly and developed blindness (Arscott and Harper, 1963). Ressler et al. (1961) found nearly three times the concentration of DABA in L. sylvestris as in L. latifolius seeds (1.4% compared to 0.51-0.67%). In a later report, Ressler (1964) noted that DABA increased dramatically on germination of L. sylvestris seeds but concentrations in hay were relatively low (seed, 0.91%; seedlings 2.55%; hay 0.02%). Conversely, moderate levels (2 to 3% dry wt.) of foliar DABA in mature flatpea have been reported by Forster (1988) and Shen (1987). Lathco flatpea DABA content varied from 0.16% in mature forage to 2.47% in mid-season regrowth (Gaffney et al., 1981). Ressler (1975) concluded that the high toxicity of L. sylvestris seed was due to an active factor (DABA) with only low to moderate toxicity (LD_{50}= 60 mg 100 mg^{-1} body weight) but present in high concentrations. Bell (1962b) identified an additional 10 species of Lathyrus containing DABA in the order of 1.0% dry wt. of seeds, but could not detect it in L. sativus, L. cicera, or L. clymenum, species which have been implicated in human lathyrism.

Some reports have suggested that feeding flatpea to sheep and other livestock can result in adverse animal effects (Daniel et al., 1946; Pavelka, 1985). Daniel et al. (1946) reported the inexplicable deaths of three sheep soon after being fed flatpea forage. The remainder of the sheep were fed flatpea for 6 months with no adverse effects. A recent study by Forster (1988) failed to substantiate toxicity to sheep. In performance and digestibility trials, a total of 80 lambs were fed different proportions of flatpea in rations also containing alfalfa and corn without any adverse effects that could be attributed to the diet. Lack of toxicity in ruminant livestock consuming flatpea forage may be due to microbial degradation of DABA in the rumen (Allison, 1978; Pavelka, 1985).

CONSERVATION AND FORAGE UTILIZATION

During a 1957 plant collection trip in the Pacific Northwest, a flatpea accession designated NY-1157 was found near Chehalis, Lewis County, WA. Seeds from this accession were planted in a small increase block at the Big Flats Plant Materials Center in 1958. No breeding or vigorous selection was done, but NY-1157 was tested against five other strains of *L. sylvestris* for plant vigor, vegetative production, seed production, seedling vigor and rate of spread (McWilliams, 1970). After extensive testing in trial plantings, NY-1157 was jointly released and the New York and Pennsylvania Agricultural Experiment Stations in 1972 under the name of Lathco. Since that time, Lathco has been planted widely as a conservation cover plant from West Virginia to Maine. Currently, flatpea is recommended for use in establishing cover on logging roads, gravel pits, utility rights-of-way, road banks, dams, and minespoil, but not for forage production (McWilliams, 1973).

In addition to the enthusiasm generated for flatpea as a conservation species, interest has revived in use of flatpea as a forage plant. After four years of harvests made at 3, 5, 10, or 15 wk intervals, stands of Lathco flatpea remained fair on the 3 wk frequency plots and good to excellent on the plots harvested at 5, 10, and 15 wk frequencies (Gaffney et al., 1981). This contrasted sharply with birdsfoot trefoil which exhibited a stand survival of only 20% on plots harvested at all clipping frequencies and crownvetch stand survival of less than 20% on plots harvested at all frequencies except 15 wk. Flatpea outyielded and contained a higher protein percentage than the other forage legumes. Humphrey et al. (1981) published a preliminary report suggesting that flatpea can be used as a permanent cover crop in no-till corn. Such utilization requires adequate suppression of established flatpea stands, which they obtained using Cyanazine, 2,4-D Ester, Glyphosate, and Atrazine plus Paraquat.

GENETIC STUDIES

Tissue Culture

Plant cell and tissue culture is now recognized as a valid adjunct to conventional breeding. These techniques offer potential for flatpea improvement through the generation of somaclonal variation, which has been defined as the induction of genetic variability in plants regenerated from cell cultures (Larkin and Scowcroft, 1981). A tissue culture cycle can result in an assortment of chromosomal changes which can provide an opportunity to screen for variant genotypes by applying selection for resistance to stress during culture (Skirvin, 1978). Somaclonal variants have been effectively recovered in tomato, tobacco, sugar cane, alfalfa and potato (Evans et al., 1984; Heinz and Mee, 1969; Pfeiffer and Bingham, 1984; Secor and Shephard, 1981). Despite these successes, the majority of somaclonal mutants are inferior to established cultivars and, in many cases, the changes are not stably inherited.

Practical application of tissue culture techniques to flatpea depends on efficient recovery of whole plants from callus tissue. Shoots and complete plants have been clonally propagated from tissue cultures of several important legumes: alfalfa (*Medicago sativa*) (Saunders and Bingham, 1972), red clover (*Trifolium pratense*) (Beach and Smith, 1979), and birdsfoot trefoil (*Lotus corniculatus*) (Tomes, 1979). Whereas no reports have been published on tissue culture of flatpea, several studies have been conducted using a relative, the grass pea, *L. sativus* L. (Gharyal and Maheshwari, 1980, 1983; Mukhopadyay and Bhojwani, 1978; Sinha et al., 1983). Shoot formation in *L. sativus* cultures was first reported by Mukhopadhyay and Bhojwani (1978) using 'LSD-6' calli derived from shoot apices. Callus was initiated on B5 medium (Gamborg et al., 1968) containing 2.85 µM indole-3-acetic acid (IAA) and 4.44 µM 6-benzylaminopurine (BAP). Although buds were easily formed, only occasional root formation was observed and shoots excised from callus failed to produce roots (Mukhopadyay and Bhojwani, 1978). Using a different cultivar of *L. sativus*, 'LSD-3', Gharyal and

Maheshwari (1980) reported consistent formation of roots in 6 to 7-week-old subcultures using B5 medium supplemented with 10.74 μM napthaleneacetic acid (NAA) and 2.22 μM BAP.

Exhaustive testing of over 200 media formulations resulted in identification of an optimum growth regulator and mineral salt combination specifically suited to in vitro culture of L. sativus (Sinha et al., 1983). The following two growth regulator combinations were found to be optimal for stem-derived tissue of L. sativus: (1) 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D) + 10.72 μM p-chlorophenoxyacetic acid (pCPA + 0.50 μM BAP, or (2) 0.50 μM trichloropicolinic acid (Picloram) + 0.50 μM BAP. Callus from 'LSD-1' L. sativus regenerated plants through shoot bud formation on the SS-B-8 medium (Sinha et al., 1983). Subsequent studies by Gharyal and Maheshwari (1983) indicated that when apical meristems of donor L. sativus were decapitated, the resulting activated axillary bud meristems produced highly morphogenic callus tissue. Altered physiological status following decapitation was thought to increase endogenous auxin concentration. Using activated axillary buds, previously noted genotype effects did not influence regeneration.

Mutation Breeding

Several breeding programs involving mutation induction have been conducted with two species of Lathyrus: tangier pea (L. tingitanus) and grass pea (L. sativus), an annual legume native to Asia. A number of mutagens including N-nitroso-N-methylurea (NMU), N-nitrosoethyl urea (NEU), ethyleneimine (EI), dimethylsulfate (DMS), ethyl methane sulphonate (EMS), 1,4-bisdiazoacetylbutane (BDAB), and gamma radiation have been utilized in mutation induction studies of L. sativus. In addition to chlorophyll mutants, a wide spectrum of viable mutations that affect plant habit, maturity, branching, stem shape, leaf size and shape, stipule shape, flower color and structure, pod size and shape and seed size and color have been detected (Chekalin, 1977a; Nerkar, 1976; Prasad and Das, 1980b;). Useful mutants included male sterile, tall plant stature, round seed, and forms with two pods per pedicel (Chekalin,

1977a). Six mutant lines of L. tingitanus were also identified which outyielded the standard check plants by 12.5 to 18.8% over three years (Chekalin, 1977a,b).

Mutation induction of tangier pea has been done exclusively with the chemical mutagens NEU, EI and DMS (Chekalin 1968, 1970; Chekalin et al., 1971; Karandasova, 1975a,b). Few differences in the spectrum of mutations induced by these compounds were found (Chekalin, 1970). Karandasova (1975a,b) reported over 40 mutations affecting morphological and physiological characters including flower color, seed color, seed shape and hypocotyl color. Twenty-two mutations were detected including such useful characters as earlier or later maturity, increased vigor, dark green leaves, absence of purple pigment in leaves and stems, and compact or dwarf habit (Chekalin, 1968). A single tangier pea mutant that contained 45% crude protein was obtained from DMS treatment (Chekalin et al., 1971). 'Poltava 2', a mutant variety obtained by repeated treatment with NEU, exceeded the yield of the standard variety 'Kharkov' by 6.7% over four years (Chekalin, 1977b).

Variation in the hard-seededness of tangier pea treated with chemical mutagens was reported by Chekalin (1970). Some mutant lines showed as low as 1.1 to 8.3% hard seeds compared with 22.4% in the original population. Breeding for soft-seededness was continued and resulted in isolation of lines that either had no hard seeds at all or that had a lower percentage of hard seeds than the original mutant (Chekalin, 1976). As a result of increased speed and uniformity of germination, dry matter yields and seed production were increased in field plantings of soft-seeded lines of tangier pea.

Nerkar (1976, 1977a,b) treated seed of five L. sativus cultivars with gamma radiation at 5, 10, 15, 20 or 30 kR, EMS at 0.15% or NMU at 0.001, 0.005 or 0.01%. Mutagenic treatments were compared for relative effectiveness, efficiency and effects on pollen and seed fertility. Rank order of the mutagens based on the frequency of mutations induced by a unit dose of mutagen was NMU > EMS > gamma radiation. Efficiency of the mutagens measured in relation to sterility and lethality was ranked gamma radiation > EMS > NMU. Both mutagenic effectiveness and

efficiency were higher at lower doses of mutagens. Although frequencies of viable and chlorophyll mutants increased with increasing dosage of gamma radiation, an inverse relationship was observed with NMU. Radiosensitivity varied considerably among cultivars. The ratio of chlorophyll to morphological mutations across all treatments in M_2 averaged 1.75:1. Gamma irradiation induced more frequent meiotic abnormalities than chemical mutagens. However, the chemical mutagens induced greater pollen and seed sterility. Pollen grains were analyzed by Prasad and Das (1980c) from M_1 and M_2 plants of six cultivars of L. sativus irradiated with 10, 20, 30, 40, 50 kR gamma radiation or treated with 0.2% MES or both. Sterility was found to vary with cultivar and increased linearly with dosage. Pollen from M_2 plants had a lower mean and variance for sterility than that from M_1 plants.

In a study to determine whether mutation induction could increase variation for β-N-oxalyl-α,β-diaminopropionic acid (ODAP) toxin content, Nerkar (1972) treated L. sativus seed of four widely grown cultivars with 5, 10 or 15 kR gamma radiation or 0.15% EMS. Seed of 20 to 50 randomly selected and selfed M_1 progenies from each treatment plus the control were screened for ODAP by paper chromatography. Heritability of ODAP content and expected genetic advance in the M_4 generation in response to selection for low-neurotoxin in the M_3 were calculated. Both gamma radiation and EMS increased the variance for ODAP from 2 to 9 fold in the M_2. A significant shift occurred in the mean toxin content and a wide range of ODAP was observed in all treatments. The four genotypes showed differential responses to the mutagens. In cultivars containing high levels of ODAP, means of ODAP were decreased and in cultivars containing low levels of ODAP, means of ODAP were increased. Heritability of low-toxin lines was estimated at 47%, based on M_3 variances. The frequency distribution in the M_2 suggested that ODAP might exhibit simple Mendelian inheritance (Nerkar, 1972).

Selection for low-ODAP content was continued in the population (Swaminathan, 1969). Low-ODAP plants bred true and were subjected to a second cycle of gamma radiation and EMS treatments to isolate mutants completely devoid of ODAP. Assessment of inheritance from the available

data indicated that this character was controlled by several genes (Swaminathan, 1969) or was polygenic (Swaminathan, 1970). Neurotoxin content gamma irradiated seeds ranged from 0.2 to 0.9%; in EMS treated seeds, from 0.2 to 0.8%; and in control seeds, from 0.5 to 0.8%. In a more recent mutation induction study in which six additional cultivars of L. sativus were treated with 10 to 50 kR gamma radiation followed by 0.2% MES treatment, Prasad and Das (1980a) isolated a few mutants in M_2 which lacked ODAP and others with reduced ODAP.

Hybridization

Since the release of Lathco flatpea in 1972, improved flatpea cultivars have not been developed. Whereas several reports have documented L. sativus breeding methods and pollination biology, flatpea crossing studies have only recently been reported. Reports by Chaib et al. (1985) have shown that considerable variation for seed production and chromosome karyotype exists in flatpea populations. Their findings also suggested that flatpea is a facultatively allogomous species. Using combinations of recessive markers, Chekalin (1972, 1973) was able to demonstrate that some cultivars of L. sativus undergo preferential fertilization by foreign pollen (60 to 100%). This trait segregated among progeny with homozygous plants exhibiting a greater tendency towards cross-pollination than heterozygous plants with respect to the marker genes. Sobolev (1966) considered that even the 15 to 25% outcrossing in L. sativus was high enough to warrant isolating accessions.

Attempts to improve flatpea through interspecific hybridization have produced little success. Crosses between Lathyrus species have often failed to produce fertile progeny, despite successful seed development after the initial cross. Early reports by Barker (1916) indicated that viable, partially fertile hybrids were obtained from the cross L. hirsutus x L. odoratus. Taylor (1916) claimed to have successfully crossed and produced seeds of L. odoratus x L. pratensis and its reciprocal, but Davies (1957) was unable to repeat this finding.

Melderis and Vikane (1931) reported unsuccessful attempts to make crosses between L. articulatus, L. magellanicus, L. odoratus, L. pratensis, L. tingitanus and L. vernus. Senn (1938) intercrossed 17 Lathyrus species and reported failure in 458 attempts including crosses and reciprocals of L. sylvestris with L. latifolius and L. heterophyllus. In four instances, seed were set but plants grown from three of the crosses proved entirely like the maternal parent in appearance, indicating likelihood of maternal pollen contamination. Marsden-Jones (1919) successfully crossed L. rotundifolius with L. tuberosus. In crossing L. odoratus x L. hirsutus, Davies (1957) employed a modification of the technique of Buchholtz et al. (1932) involving complete amputation of the female parent style followed by pollination on the cut stump. Partial stylar removal resulted in occasional fertilizations. Grafting of L. hirsutus styles onto ovaries of L. odoratus showed no advantage over direct pollination on the cut stump (Davies, 1957). Of 26 interspecific crosses made by Brown and Williams (1965), only six produced a small quantity of viable seeds. All successful crosses were obtained from plants treated with the hormone α-naphthylacetamide in lanolin.

Embryos of the cross L. clymenum x L. articulatus that aborted in vivo at 0.5-1.0 mm size were rescued by excision just before abortion and placement into an inorganic medium amended with thiamine, nicotinic acid, 12% (w/v) sucrose and 10% (v/v) coconut milk (Peckett and Selim, 1963). A high percentage of the embryos subsequently transferred at 5 d onto a medium containing 4% sucrose and no coconut milk germinated within 10 days. After occasional transfers into fresh tubes of medium containing 4% sucrose and 50 mg L^{-1} glutamine over 6 wk, young plants were potted in soil.

Failure of some Lathyrus species to hybridize may be related to their amino acid composition. Bell (1964, 1965) classified 53 Lathyrus species into five biochemical and taxonomic groupings based on the pattern of amino acids and related compounds isolated from seed extracts. Lathyrus species showing dissimilar amino acid patterns have not produced viable hybrids (Bell and Fowden, 1964).

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Chapter III

FLATPEA ORGANOGENESIS FROM CALLUS CULTURES AND FREE AMINO ACID COMPOSITION OF REGENERATED TISSUES

ABSTRACT

Flatpea (Lathyrus sylvestris L.) is a potentially valuable forage legume, but it contains high levels of 2,4-diaminobutyric acid (DABA), a compound that can have adverse effects on some animals, including rats and poultry. Regeneration of flatpea plants from tissue culture was attempted to produce somaclonal variation for flatpea traits such as DABA content. Flatpea tissues were evaluated for regeneration potential on several media formulations. Low-frequency whole plant regeneration of flatpea was obtained from hypocotyl-derived callus cultures. Auxin concentrations above 5.0 µM resulted primarily in root formation without shoots. Addition of IAA amino acid conjugates was less effective than free IAA in producing organogenesis. Conditions for optimum whole plant regeneration were as follows: callus was initiated on a modified Schenk-Hildebrandt low-ammonium medium containing 5.0 µM IAA and 2.5 µM zeatin for callus initiation; after two subcultures, calli were transferred to basal medium containing 1.0 µM zeatin and 0.1 µM (IAA). Root and shoot primordia developed approximately 60 d after explant transfer to callus induction medium. The optimized regeneration protocol developed in this study should facilitate further genetic improvement of flatpea using in vitro techniques.

INTRODUCTION

Flatpea (Lathyrus sylvestris L.) has several advantages as a forage and conservation legume, including persistence on low-fertility soils and tolerance to environmental stresses. However, utilization of flatpea has been limited due to the possibility of detrimental effects on some animals consuming flatpea forage. Adverse effects of flatpea consumption have been observed in rats and poultry (Huang et al., 1950; Miller, 1946) and Ressler (1961) has identified 2,4-diaminobutyric acid (DABA) as a neuroactive component of flatpea. Selection of flatpea for traits of interest has been hampered because of insufficient genetic variability. A single flatpea cultivar, 'Lathco', released in 1972 by the Soil Conservation Service, is commercially available.

Somaclonal variation is now recognized as an alternative method of inducing variability through plant regeneration from cell and tissue cultures (Evans et al., 1984; Larkin and Scowcroft, 1981; Skirvin, 1978). Additional tissue culture manipulations, such as in vitro selection, that supplement conventional breeding also require establishment of conditions for effective callus induction and organogenesis. Culture conditions necessary for callus induction and plant regeneration of flatpea have not been reported. Formation of complete plants from in vitro cultures has been demonstrated for Lathyrus sativus L., a relative of the flatpea (Gharyal and Maheshwari, 1980; Gharyal and Maheshwari, 1983; Mukhopadhyay and Bhojwani, 1978). Objectives of this investigation were: (1) to optimize an auxin source for callus growth, identify responsive flatpea accessions, determine proper explants, and refine a tissue culture medium for flatpea plant regeneration and (2) to determine the DABA and free amino acid composition of the plant material produced.

MATERIALS AND METHODS

Callus Growth Evaluation Using Different Auxin Sources

In a preliminary test, 2,4-dichlorophenoxyacetic acid (2,4-D) and napthaleneacetic acid (NAA) were compared for ability to stimulate callus production using stem-derived flatpea explants. For these comparisons, Lathco flatpea source plants were maintained in a walk-in controlled environment growth chamber programmed for an 18 h light/6 h dark cycle, 25°C, and 60% relative humidity. Cool white fluorescent lamps produced an average illumination of 550 µmol m^{-2} s^{-1} at canopy height. Plants were grown in 11.4 L-capacity pots and supported with woven wire (5.1 x 10.2 cm mesh) cylindrical trellises. Weekly fertilizer additions consisted of 2.2 g L^{-1} 20-20-20 liquid fertilizer.

Stem tissue used for callus growth originated from internode sections of 20-wk-old flatpea plants that had been cut at the crown level and allowed to regrow for 3 wk. Excised stems were surface-sterilized by 5-min immersion in 70% (v/v) aqueous ethanol, followed by 5 min in 50% (v/v) aqueous commercial bleach containing a drop of 'Tween 20'. After three rinses in sterile water, stems were dissected into 8-mm transverse sections.

Each auxin was evaluated at 10, 25, and 50 µM concentrations using Murashige Skoog (1962, hereafter MS) medium containing 10 µM 6-benzylaminopurine (BAP). MS medium contained 30 g L^{-1} sucrose, and 7.0 g L^{-1} phytagar; pH was adjusted to 5.75 before autoclaving. Each explant was placed in a separate 25 x 125 mm culture tube containing 20 ml of MS medium. Four replications of each treatment (ten explants per replicate) were completely randomized in the growth chamber. Cultured explants were incubated in a growth chamber with a 16 h light/8 h dark cycle at 25°C. Cool white fluorescent lamps provided an average illumination of 35 µmol m^{-2} s^{-1}. After 3 months in culture, calli were cleaned of any adhering agar and weighed.

Accession Screening for Organogenesis

Accessions used in this study were provided by Dr. F. Bisby, University of Manchester, United Kingdom. In addition to the U.S. cultivar, Lathco, the following British accession numbers were evaluated (country of origin indicated in parentheses): 780091, 780567, 780670 (U.S.S.R.); 780099, 780100 (East Germany); 780201 (United Kingdom); 780601 (Ireland). Growth conditions for source plants and cultures were as described for callus growth evaluation. Flatpea accessions were screened for regeneration potential using stem-derived explants and a modified Schenk-Hildebrandt (1972, hereafter SH) medium supplemented with a combination of 25 μM NAA and 10 μM BAP. Due to the reported sensitivity of flatpea to ammonium ions when grown in nutrient solutions (Shen, 1987), SH medium was modified by lowering the NH_4NO_3 concentration to 150 mg L^{-1} and designated SH-LA (Schenk-Hildebrandt low-ammonia). Six replications of 40 explants per replicate in 15 x 100 mm petri plates were used to compare accessions. Each replication was derived from a separate plant. Whole pieces of callus were subcultured 28 d after culture initiation by transfer to a fresh callus induction medium. When observed, necrotic bases of calli were removed with a scalpel and only green sections were transferred. After a second 28 d period on callus induction medium (25 μM NAA and 10 μM BAP), calli were transferred to a regeneration medium containing 0.1 μM indoleacetic acid (IAA) and 1.0 μM 6-[4-hydroxy-3-methylbut-2-enyl-amino] purine (zeatin). Proportions of plated explants producing roots, shoots, or both were recorded for each petri plate. In cases where roots formed from a different part of callus than shoots, cultures were counted as forming roots and shoots separately, without whole plant regeneration.

Determination of Organogenic Potential of Flatpea Explants

Explants were obtained from seedling hypocotyls, ovaries, and stem tissue of mature plants. Hypocotyl explants were obtained from Lathco flatpea seeds (Big Flats Plant Materials Center, Corning, NY, U.S.A., lot no. LASY81FR) which had been scarified by soaking in concentrated H_2SO_4 for 1 min followed by thorough rinsing with sterile water. Air-dried, scarified seeds were then disinfested by immersing in concentrated bleach for 15 min on a rotary shaker. After thorough rinsing with sterile water under a laminar transfer hood, seeds were aseptically transferred to 25 X 125 mm culture tubes containing 20 ml agar-solidified Schenk-Hildebrandt (SH) nutrient medium. Hypocotyls were excised from seedlings 14 d after transfer to the medium, cut into 8-mm transverse sections, and placed on SH medium supplemented with 25 µM NAA and 10 µM BAP.

Ovary tissue was collected from unfertilized flowers at late-bud stage. Flowers were soaked for one min in 95% (v/v) aqueous ethanol followed by one min in 20% (v/v) aqueous commercial bleach solution containing a drop of 'Tween 20' as a wetting agent. After rinsing three times in sterile water, flowers were immediately dissected. All manipulations were carried out under aseptic conditions. Each ovary was slit longitudinally and the cut surface was placed in contact with the growth medium. Each explant was placed in a separate 25 x 125 mm culture tube containing 20 ml of callus induction medium as described for hypocotyls. Stem tissue preparation and subculture protocol were as described for accession screening.

Media Refinements for Increasing Organogenic Potential of Flatpea Accession 780901-C

Growth Regulator Evaluation. Plant growth regulators that were evaluated included IAA, NAA, 2,4-D, BAP, N^6-(^{2}-isopentenyl) adenine (2iP), 6-furfurylaminopurine (kinetin), and zeatin. The following IAA-amino acid conjugates were provided by Dr. Norman Good at Michigan State University: indoleacetyl aspartate (IAA-Asp), indoleacetyl alanine (IAA-Ala), and indoleacetyl glycine (IAA-Gly). Additions of free IAA to callus induction media is sometimes ineffective due to enzymatic degradation of IAA over time. Initial experiments indicated poor callus growth in long term cultures utilizing free IAA. Conjugates were therefore added to SH-LA medium as slow release forms of free IAA. Growth regulators were filter-sterilized using 0.20 µm Acrodisc (Fisher Scientific, Pittsburgh, PA, U.S.A.) filters. IAA, IAA-conjugates, and NAA were compared at 2.5 and 5.0 µM levels of zeatin using Lathco flatpea stem explants. Auxin levels were supplied at 5, 10, 15, 20, and 25 µM using SH-LA.

Zeatin, 2iP, and kinetin were each evaluated at 2.5 and 5.0 µM levels. These treatment levels were applied to hypocotyl-derived explants of accession 780901-C that were cultured on SH-LA nutrient medium containing 5.0 µM IAA.

Media Evaluation. Media formulations that were evaluated included SS-B-8 (Sinha et al., 1983), MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), and SH (Schenk and Hildebrandt, 1972). Comparisons were made among media with hypocotyl-derived explants from accession 780901-C. All media were supplemented with 5.0 µM IAA and 2.5 µM zeatin. Additives tested included 0.4 g L^{-1} casein hydrolysate, 0.4 g L^{-1} glycine, 1.0 g L^{-1} coconut milk, and 1.0 g L^{-1} yeast extract. Coconut milk was heated for 30 min at 60°C and filtered through a Whatman no. 2 filter.

Nonprotein Amino Acid Analysis

Calli, regenerated roots, or fully-expanded, deep green mature leaves of regenerated whole plants were crushed in liquid nitrogen using a mortar and pestle and lyophilized. For analysis of free amino acids, 0.2 to 0.4 g of lyophilized tissue was extracted for 90 min using 90 ml of 50% (v/v) aqueous ethanol in 30 mm i.d. Soxhlet extraction tubes. Two ml of 15 mM S-(4-pyridylethyl)-DL-penicillamine (Pierce Chemical Co.. Rockford, IL, U.S.A.) was added as an internal standard. Sample extracts were concentrated under nitrogen at 40°C; the resulting residue was resuspended in 10 ml of extraction medium. A 2.5 ml aliquot of this suspension was centrifuged at 3,000 g for 10 min. The supernatant was brought to a total volume of 7.5 ml with extraction medium; 0.25 ml of this preparation was loaded onto a Sep-Pak C_{18} column (Waters Associates, Milford, MA, U.S.A.) and eluted successively with 0.5 ml of water and 1.0 ml of methanol. Combined eluates were adjusted to a final volume of 2.0 ml with water.

Free amino acids in extracts were derivatized with o-phthalaldehyde (OPA, Pierce Chemical Co.) prepared by dissolving 50 mg of OPA in 1 ml of HPLC-grade methanol, adding 50 µL of 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and bringing the solution to a final volume of 10 ml with 0.40 M sodium borate-KOH, pH 9.5 containing 0.1% (v/v) Brij 35 (polyoxyethylene lauryl ether, Fisher Scientific, Pittsburgh, PA, U.S.A.). Freshly prepared OPA stock solution was stored overnight at 0 to 5°C before use and was used for 2 d. A 0.1 ml aliquot of the solution was mixed with 0.02 ml of extract 90 s before injection onto the HPLC column. Derivatized samples were analyzed using a Beckman model 344 binary gradient HPLC system equipped with an Altex 4.6 x 45 mm, 5 µm Ultrasphere-ODS octadecylsilane precolumn and an Altex 4.6 x 250 mm, 5 µm Ultrasphere-ODS octadecylsilane analytical column maintained at 45°C.

Amino acid derivatives were detected using a Gilson model 121 fluorescence detector equipped with a 9 µL flow cell and filters for excitation at 305 and 395 nm and emission at 430 to 470 nm. Detector

range and time constant settings were 0.02 relative fluorescence units and 0.5 s, respectively. Amino acids in tissue extracts were identified by comparison of their retention times to those of pure amino acid standards (Sigma Chemical Co.) and by coinjection of samples and the standards. Peak areas were determined using a Nelson Analytical (Cupertino, CA, U.S.A.) model 4416X chromatography data system. Amino acids were quantified using standard curves and yields of individual amino acids in extracts were calculated based on recoveries of the internal standard.

Statistical Analysis

Culture tubes and petri plates for all experiments were completely randomized in the growth chamber. Number of cultures producing roots, shoots, or whole plant regeneration did not fit a normal distribution and differences between treatment means were therefore analyzed using a chi-square goodness of fit test for equality of the proportions of cultures responding. Individual mean comparisons were performed using the Bonferoni inequality to produce a level of significance that guarantees an overall type I error rate of no more than 0.05. Analysis of variance was performed on DABA concentration of flatpea tissues and DABA means for different tissues were compared using Fisher's Least Significant Difference (LSD) multiple range test. A SAS general linear models (GLM) regression analysis was used to test the relationship between auxin level and callus growth.

RESULTS AND DISCUSSION

Callus Growth Evaluation Using Different Auxin Sources

Callus production was markedly inhibited by 2,4-D over a concentration range of 10 to 50 µM (Fig. 1). NAA produced greater callus fresh weights than 2,4-D and was most effective when supplied at 10 to 25 µM.

Accession Screening for Organogenesis

Comparisons among European and Asian flatpea accessions under uniform conditions of 25 µM NAA and 10 µM BAP resulted in identification of high regenerator flatpea genotypes (Table 1). An individual genotype of accession 780901 (designated 780901-C), originating from the U.S.S.R., produced whole plant regeneration and significantly higher caulogenesis than other accessions. Although less organogenic than 780901-C in rhizogenesis or caulogenesis, accessions 780201 and 780670 also produced whole plants. Lathco stem explants failed to regenerate whole plants and exhibited significantly lower rhizogenesis or caulogenesis than 780901. Regeneration was typically evident during the first or second transfer onto regeneration medium (following two 28-d culture periods on callus induction media).

Determination of Organogenic Potential of Flatpea Explants

Hypocotyl explants, obtained from a high regenerator plant identified from accession 780901, exhibited significantly greater rhizogenesis or whole plant regeneration than those obtained from stem or ovary tissue (Table 2). Explants from stem tissue produced both roots and shoots, but did not result in whole plant regeneration. Although ovary tissue was the least organogenic of the explants evaluated, it produced an extremely friable callus, which may be suitable for flatpea suspension cultures.

Media Refinements for Increasing Organogenic Potential of Flatpea Accession 780901-C

Growth Regulator Evaluation. At either 2.5 or 5.0 µM zeatin, rhizogenesis was the primary response to increasing levels of IAA or NAA in the callus induction medium (Table 3). Because shoot and whole plant regeneration were negligible using Lathco flatpea explants and these growth regulators, only rhizogenesis is reported in Table 3. Except at the highest level of auxin, NAA promoted higher frequencies of root formation than IAA. Rhizogenic cultures of this type were usually undesirable due to their inability to form shoots. In subsequent experiments, auxin levels were reduced to 5 µM or less to encourage caulogenesis. In all cases where significant differences were found within auxin treatment levels, IAA-Gly significantly lowered root production compared to the free auxins. Root formation was generally inhibited by IAA-amino acid conjugates compared to the free auxins, although few significant differences were found among auxin sources at the 5, 10, and 15 µM treatment levels (Table 3).

IAA-amino acid conjugates that effectively release auxin at a slow rate were generally not beneficial for whole plant regeneration in this study. However, suppression of rhizogenesis by IAA-conjugates at certain concentrations may be desirable for cultures that produce roots only and do not respond to shoot induction media. Conjugates have been reported to reduce in vitro root initiation in other plant species (Hangarter et al., 1980). Above the 15 µM treatment level, IAA-Asp was generally less effective in reducing root formation than the other IAA-conjugates, except at the 25 µM level at 5.0 µM zeatin. Failure of IAA-Asp to diminish rooting in these cases may be related to its content of the less active D-stereoisomer (N.E. Good, 1985, personal communication).

Cytokinin responses (Table 4) varied according to the compound and concentration. Whole plant regeneration was higher for all cytokinin sources at the 2.5 µM level than at 5.0 µM. Conversely, incomplete plant regeneration (rhizogenesis or caulogenesis) from callus was

generally higher at the 5.0 μM cytokinin treatment level. Additions of 5.0 μM kinetin to the regeneration medium resulted in the highest overall percentage of root production, whereas 2.5 μM zeatin produced the highest whole plant regeneration. Zeatin and BAP were equally effective in promoting shoot production. Regenerated shoots were typically weak and failed to differentiate roots when placed on a root induction medium containing 0.1 μM IAA and no cytokinin.

Media Evaluation. Evaluations of standard nutrient media formulations (Table 5) indicated that both B5 and SH-LA media promoted a high degree of organogenesis. Additives generally diminished the effectiveness of SH-LA medium in rhizogenesis and caulogenesis, but SH-LA plus casein hydrolysate was equal to SH-LA for whole plant regeneration. SS-B-8 medium was the least effective mineral salt formulation tested, although SS-B-8 was used successfully in regeneration of L. sativus cultures (Sinha et al., 1983).

The most effective protocol for whole plant regeneration identified in this study involved culturing hypocotyl explants for two 28-d subculture periods on SH-LA callus induction medium containing 5.0 μM IAA or NAA and 2.5 μM zeatin. Callus was then transferred to regeneration media containing 1.0 μM zeatin and 0.1 μM IAA.

Regeneration potential in flatpea appears to be under genetic control, although enhancement of organ differentiation is possible through alterations in growth regulator composition of the medium. Early studies utilizing L. sativus tissue cultures indicated that shoots were produced more readily than roots (Mukhopadhyay and Bhojwani, 1978). In contrast, root formation without shoots was the predominant regenerative response in flatpea cultures.

Nonprotein Amino Acid Analysis

Wide differences in amino acid composition among callus, regenerated roots, and leaves from regenerated plants were observed (Table 6). Although regenerated plants did not show substantial reduction in DABA concentration, calli and regenerated root tissue contained 47 and 38% as much foliar DABA as Lathco flatpea, respectively. Based on the few regenerated plants available for analysis, variation in foliar DABA concentration was comparable to the range of values measured in flatpea accessions. In most cases, amino acid concentrations were higher in callus or regenerated shoots than whole plant foliar levels when expressed on a dry wt. basis.

Results of studies addressing the intracellular localization of DABA in leaf mesophyll cells were interpreted as evidence for chloroplastic synthesis and vacuolar storage of DABA in those cells (Foster, et al., 1987). In the present study, DABA concentrations in callus and regenerated roots suggest the presence of synthetic enzymes in nonchlorophyllous tissue. Occurrence of moderate levels of DABA in regenerated roots implicates an alternative site for DABA synthesis in addition to the chloroplast. Concentration of DABA in callus could not be solely attributed to DABA content of the original explant. Callus and regenerated root concentrations of nonprotein amino acids, DABA and GABA in Table 6, were similar to those reported by Shen (1987) for whole plant roots. Lower DABA levels in flatpea callus, compared to those routinely observed in leaf tissue from whole plants, is noteworthy because of the possibility for selection of low-DABA cell lines and regeneration of low-DABA plants. However, reduced DABA production in callus cell lines may be unrelated to DABA produced by whole plant leaves because a primary site of DABA synthesis in flatpea appears to be the chloroplast (Foster et al., 1987).

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Table 1. Regeneration frequencies of stem explants from different flatpea accessions after callus initiation on Schenk-Hildebrandt low-ammonia (SH-LA) medium containing 25 μM NAA and 10 μM BAP.[a]

Accession	Type of regeneration				
	Rhizogenesis		Caulogenesis		Whole plant regeneration
	no. of cultures producing roots per 100 explants		no. of cultures producing shoots per 100 explants		no. of cultures producing whole plants per 100 explants
780901	47.6[b]	a	5.9	a	0.9
780099	32.1	b	0.4	cd	0.0
780567	28.5	b	1.3	bc	0.0
780201	13.6	c	3.2	ab	0.5
780670	11.3	c	0.0	d	0.6
Lathco	10.4	cd	1.4	bc	0.0
780601	6.6	de	0.0	d	0.0
780100	4.9	e	0.0	d	0.0
Chi-square	*		*		NS

NS not significant

[a]Means represent six replications (40 explants per replicate); NAA = Napthaleneacetic acid; BAP = 6-Benzylaminopurine.

[b]Values within a column followed by the same letter are not significantly different (Bonferoni test for inequality of proportions);

* Significant chi-square value at 5% level testing the inequality of proportions.

Table 2. Regeneration frequencies of flatpea callus cultures using different explant sources after callus initiation on Schenk-Hildebrandt low-ammonia (SH-LA) medium containing 25 μM NAA and 10 μM BAP[a]

Explant	Type of regeneration: Rhizogenesis	Caulogenesis	Whole plant regeneration
	no. of cultures producing roots per 100 explants	no. of cultures producing shoots per 100 explants	no. of cultures producing whole plants per 100 explants
Hypocotyl	71.5[b] a	5.6	8.7 a
Stem	12.5 b	5.0	0.0 b
Ovary	6.2 b	0.0	0.0 b
Chi-square	***	NS	*

[a]Explants were obtained from accession 780901-C; means represent three replications (10 explants per replicate); NAA = Napthaleneacetic acid; BAP = 6-Benzylaminopurine.

[b]Values within a column followed by the same letter are not significantly different (Bonferoni test for inequality of proportions).

*,*** Significant chi-square values at 5% and 0.1% levels testing the inequality of proportions.

Table 3. Frequencies of rhizogenesis of flatpea callus cultures initiated on Schenk-Hildebrandt low-ammonia (SH-LA) medium containing free and conjugated auxins.[a]

Auxin	Zeatin	Auxin concentration (µM)					Trend	
		5	10	15	20	25	Linear	Quad
	(µM)	no. of cultures producing roots per 100 explants[b]						
IAA	2.5	8.3	13.3	28.6	25.3 b	53.5	**	NS
IAA-Gly	2.5	5.3	11.1	18.1	5.3 c	5.0	NS	NS
IAA-Asp	2.5	10.6	17.5	20.0	26.9 b	25.0	NS	NS
IAA-Ala	2.5	5.0	17.8	20.2	20.0 b	7.8	NS	**
NAA	2.5	15.6	33.3	34.1	61.7 a	46.2	**	NS
Chi-square		NS	NS	NS	***	***		
IAA	5.0	5.0	24.4 b	31.2	22.4 b	62.0 a	**	NS
IAA-Gly	5.0	0.0	8.1 c	13.8	2.8 c	18.1 b	NS	NS
IAA-Asp	5.0	5.3	10.3 c	19.9	16.8 b	16.4 b	NS	NS
IAA-Ala	5.0	3.6	16.6 bc	18.8	5.0 c	18.9 b	NS	NS
NAA	5.0	5.3	69.1 a	40.0	52.8 a	28.2 b	NS	**
Chi-square		NS	***	NS	***	***		

** F value significant at the 1% level.
NS not significant
[a]Stem-derived explants were obtained from Lathco flatpea; means are an average of three replications (10 explants per replicate).
[b]Values within a column followed by the same letter are not significantly different (Bonferoni test for inequality of proportions).
*, *** Significant chi-square values at 5% and 0.1% levels testing the inequality of proportions.
IAA = indoleacetic acid; NAA = napthaleneacetic acid
Zeatin = 6-[4-hydroxy-3-methylbut-2-enyl-amino] purine

Table 4. Regeneration frequencies of flatpea callus cultures initiated on Schenk-Hildebrandt low-ammonia (SH-LA) medium containing different cytokinin sources and 5.0 µM IAA.[a]

Cytokinin	Zeatin	Type of regeneration: Rhizogenesis		Caulogenesis		Whole plant regeneration
	(µM)	no. of cultures producing roots per 100 explants[b]		no. of cultures producing shoots per 100 explants		no. of cultures producing whole plants per 100 explants
BAP	2.5	2.5		2.5		5.6
Zeatin	2.5	5.0		2.5		15.0
2iP	2.5	2.8		0.0		5.0
Kinetin	2.5	5.6		0.0		5.0
Chi-square		NS		NS		NS
BAP	5.0	10.0	b	19.4	a	0.0
Zeatin	5.0	18.0	ab	18.2	ab	2.8
2iP	5.0	2.5	c	0.0	c	3.1
Kinetin	5.0	31.1	a	7.8	b	0.0
Chi-square		*		*		NS

[a]Hypocotyl-derived explants were obtained from flatpea accession 780901-C; means represent three replications (10 explants per replicate); IAA = Indoleacetic acid.

[b]Values within a column followed by the same letter are not significantly different (Bonferoni test for inequality of proportions).

*,*** Significant chi-square values at 5% and 0.1% levels testing the inequality of proportions.

BAP = 6-benzylaminopurine

Zeatin = 6-[4-hydroxy-3-methylbut-2-enyl-amino] purine

2iP = N^6-(2-isopentenyl) adenine; kinetin = 6-furfurylaminopurine

Table 5. Regeneration frequencies of flatpea callus cultures initiated on different nutrient media containing 5.0 µM IAA and 2.5 µM zeatin.[a]

Medium[c]	Type of regeneration: Rhizogenesis		Caulogenesis		Whole plant regeneration
	no. of cultures producing roots per 100 explants		no. of cultures producing shoots per 100 explants		no. of cultures producing whole plants per 100 explants
SS-B-8	5.6[b]	f	0.0	c	0.0
MS	41.9	bc	2.8	bc	0.0
B5	61.3	ab	16.2	a	5.6
SH-LA	62.8	a	19.3	a	7.8
SH-LA + Gly	9.2	ef	3.6	bc	3.6
SH-LA + Pro	20.0	de	5.0	bc	0.0
SH-LA + CH	33.3	cd	7.8	ab	7.8
SH-LA + CM	38.5	c	9.0	a	0.0
Chi-square	***		*		NS

[a]Hypocotyl-derived callus was obtained from flatpea accession 780901-C; means represent three replications (10 explants per replicate); IAA = Indoleacetic acid; Zeatin = 6-[4-hydroxy-3-methylbut-2-enyl-amino] purine.

[b]Values within a column followed by the same letter are not significantly different (Bonferoni test for inequality of proportions).

*,*** Significant chi-square values at 5% and 0.1% levels testing the inequality of proportions.

[c]SS-B-8 (Sinha et al., 1983); MS = Murashige Skoog; B5 = Gamborg B5; SHLA = Schenk-Hildebrandt low-ammonia; CH = casein hydrolysate; CM = coconut milk.

Table 6. Nonprotein amino acid composition of flatpea tissue derived from hypocotyl explants cultured on Schenk-Hildebrandt low-ammonia (SH-LA) nutrient medium containing 5.0 μM IAA and 2.5 μM zeatin.[a]

Amino acid	Plant tissue			
	Callus	Regenerated roots	Regenerated whole plant mature leaves	Reported values for whole plant roots[c]
	------------------ mg g^{-1} dry wt. ------------------			
DABA	20.2 ± 4.1[b]	16.1 ± 5.2	33.8 ± 21.2	16.7 ± 1.7
GABA	22.8 ± 4.4	43.4 ± 21.9	45.3 ± 14.4	17.1 ± 1.8

[a]Means represent three plants; IAA = Indoleacetic acid; Zeatin = 6-[4-hydroxy-3-methylbut-2-enyl-amino] purine.

[b]± SE

[c]Values were reported by Shen (1987); means represent 18 plants averaged over three harvest dates, drought stressed, and control plants grown for 15 wk in a Lily soil.

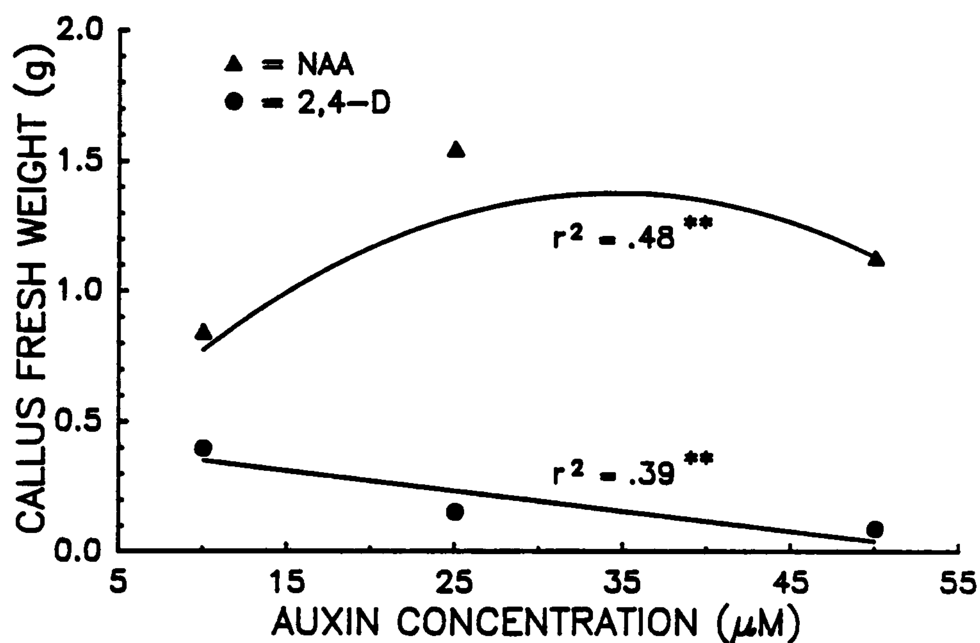

Fig. 1. Callus growth of Lathco flatpea stem explants after 12 wk growth on Murashige Skoog (MS) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and napthaleneacetic acid (NAA). Each regression point is the mean of at least seven calli. Regression equations: callus fresh wt growth (mg) = $102.5\ x - 1.591\ x^2 - 21.16$ (NAA); callus fresh wt growth (mg) = $-28.01\ x + 642.9$ (2,4-D).

Chapter IV

INITIAL STUDIES ON THE MUTAGENESIS OF FLATPEA GERMPLASM USING SEED IRRADIATION

ABSTRACT

Flatpea (Lathyrus sylvestris L.) is a potentially valuable forage legume, but it contains high levels of 2,4-diaminobutyric acid (DABA), a compound that can have adverse effects on some animals, including rats and poultry. 'Lathco' flatpea seeds were exposed to gamma radiation in order to increase genetic variability in DABA content and other desired traits. Preliminary tests established that the effective range of gamma radiation was between 10.0 and 17.5 kR. Within this range, reduction in percentage of both seedling height and plant survival was a linear function of dose. Low numbers of chlorophyll and morphological mutants were observed in the M_2. Individual M_2 plants that contained reduced levels of DABA were identified by analysis of amino acid composition of mature leaves. M_2 plants were found with as low as 6.6 mg g^{-1} dry wt. foliar DABA compared to a minimum value of 17.3 mg g^{-1} dry wt. foliar DABA in nonirradiated plants. No significant trend in DABA concentration with increasing gamma radiation dose was apparent.

INTRODUCTION

Flatpea (Lathyrus sylvestris L.) has several advantages as a forage and conservation legume for cool, temperate climates, including persistence on low-fertility soils and tolerance to environmental stresses. However, utilization of flatpea has been limited due to the possibility of detrimental effects on some animals consuming flatpea forage. Adverse effects of flatpea consumption have been observed in rats and poultry (Huang et al., 1950; Miller, 1946) and Ressler (1961) has identified 2,4-diaminobutyric acid (DABA) as a neuroactive component of flatpea. Selection of flatpea for traits of interest has been hampered

because of insufficient genetic variability. A single flatpea cultivar, 'Lathco', released in 1972 by the Soil Conservation Service, is commercially available.

Lack of previous genetic studies using flatpea and a narrow genetic base in Lathco flatpea populations prompted a mutation induction program designed to increase variability in a number of traits including DABA content. Mutation breeding has been used extensively in a related species, Lathyrus sativus L., to reduce plant levels of the neurotoxin, 3-N-oxalyl-2,3-diaminopropionic acid (ODAP, also referred to as β-oxalyl amino-L-alanine, or BOAA) (Chekalin, 1977; Nerkar, 1972, 1976, 1977; Prasad and Das, 1980). Nerkar (1972) treated L. sativus seed of four cultivars with 5, 10, or 15 kR gamma radiation or 0.15% (w/v) ethyl methane sulphonate (EMS). Both gamma irradiation and EMS increased the variance for ODAP content from 2 to 9 fold in the M_2. Using 10 to 50 kR gamma radiation followed by 0.2% EMS treatment, Prasad and Das (1980) isolated mutants in the M_2 with reduced ODAP, including some mutants devoid of ODAP.

Chekalin (1970) reported reduction in hard-seededness of Lathyrus tingitanus L. using N-nitrosoethyl urea (NEU), ethyleneimine (EI) and dimethylsulfate (DMS). Some mutant lines showed as low as 1.1 to 8.3% hard seeds compared with 22.4% in the original population. Breeding for soft-seededness was continued and resulted in isolation of lines that either had no hard seeds or that had a lower percentage of hard seeds than the original mutant (Chekalin, 1976).

Mutation breeding has also been effective in reducing coumarin content of sweet clover Melilotus alba (Micks, 1969) and alkaloid content of lupins (Lupinus mutabilis) (Pakendorf and Rensburg, 1981). Similar success using flatpea mutagenesis may prove more difficult because self-pollinated species such as L. sativus L. are considered more suitable for mutation studies than the naturally cross-pollinated flatpea. This paper reports on initial studies of flatpea mutagenesis including sensitivity of flatpea to gamma irradiation and early generation screening for DABA concentration.

MATERIALS AND METHODS

Lathyrus sylvestris L. 'Lathco' seeds (Big Flats Plant Materials Center, Corning, NY, U.S.A., lot no. LASY81FR) were acid scarified by immersion in concentrated H_2SO_4 for 10 min, rinsed three times in distilled water, and air dried to 10.5% moisture prior to radiation treatment. Results of a preliminary trial indicated that seed exposure to 20 or 30 kR gamma radiation was lethal to seedling growth. Only the 10 kR treatment permitted continued growth following germination. Based on these findings, an effective treatment range of 10.0 to 17.5 kR was used. Seeds were exposed to 10.0, 12.5, 15.0, or 17.5 kR gamma radiation at a dose rate of 2.5 kR min^{-1} from a ^{60}Co source contained in a Model GR12-D Irradiator. Seed treatment was conducted at the Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana. A ferrous sulfate chemical dosimeter was used to calibrate the source (Dr. S. Shaw, 1986, personal communication). Fifty seeds from each treatment (including the nonirradiated control) were germinated in 15 x 100 mm petri plates. Germinated seeds were planted in rows that were randomized within a flat containing a peat moss and perlite potting mixture and timed release 17-6-12 fertilizer plus micronutrients (Sierra Chemical Co., Milpitas, CA, U.S.A.) without rhizobia inoculation. Five replications were used, each consisting of a single flat. Seedlings were grown in a walk-in growth chamber programmed for a 16 h light/8 h dark cycle, 25°C, and 60% relative humidity. An average illumination of 550 μmol m^{-2} s^{-1} was produced by cool white fluorescent and incandescent lamps. Three wk after planting, seedling height was measured from soil level to plant tops and percentage of surviving seedlings was recorded. Twenty surviving seedlings per treatment were transplanted into 15.2-cm pots containing a potting mixture as described for seedling survival tests. Plants were grown to maturity in the greenhouse with average temperatures of 24±2°C (day) and 19±2°C (night). The period of illumination was extended to 18 h using high pressure sodium lamps. Plants received weekly fertilizer additions of 2.2 g L^{-1} 20-20-20 liquid fertilizer. After 4 wk of growth, M_1

plants were transferred to 11.4 L capacity pots and were supported with woven wire (5.1 x 10.2 cm mesh) cylindrical trellises. Opened flowers were hand-tripped using the abraded surface of a microscope slide for self-pollination. Selfed seed from these plants was bulked and a random sample of 60 seeds per treatment was planted in 15.2-cm pots, with one plant per pot. After 2 months of growth in the greenhouse, six randomly selected M_2 plants per treatment were analyzed individually for amino acid composition according to the method of Foster et al. (1987). Fully-expanded, deep green mature leaves were harvested, immersed in liquid nitrogen, and crushed using a mortar and pestle.

For analysis of free amino acids, 0.2 to 0.4 g of lyophilized tissue was extracted for 90 min using 90 ml of 50% (v/v) aqueous ethanol in 30 mm i.d. Soxhlet extraction tubes. Two ml of 15 mM S-(4-pyridylethyl)-DL-penicillamine (Pierce Chemical Co.. Rockford, IL, U.S.A.) was added as an internal standard. Sample extracts were concentrated under nitrogen at 40°C; the resulting residue was resuspended in 10 ml of extraction medium. A 2.5 ml aliquot of this suspension was centrifuged at 3,000 g for 10 min. The supernatant was brought to a total volume of 7.5 ml with extraction medium; 0.25 ml of this preparation was loaded onto a Sep-Pak C_{18} column (Waters Associates, Milford, MA, U.S.A.) and eluted successively with 0.5 ml of water and 1.0 ml of methanol. Combined eluates were adjusted to a final volume of 2.0 ml with water.

Free amino acids in extracts were derivatized with o-phthalaldehyde (OPA, Pierce Chemical Co.) prepared by dissolving 50 mg of OPA in 1 ml of HPLC-grade methanol, adding 50 µL of 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and bringing the solution to a final volume of 10 ml with 0.40 M sodium borate-KOH, pH 9.5 containing 0.1% (v/v) Brij 35 (polyoxyethylene lauryl ether, Fisher Scientific, Pittsburgh, PA, U.S.A.). Freshly prepared OPA stock solution was stored overnight at 0 to 5°C before use and was used for 2 d. A 0.1 ml aliquot of the solution was mixed with 0.02 ml of extract 90 s before injection onto the HPLC column. Derivatized samples were analyzed using a Beckman model 344 binary gradient HPLC system equipped with an Altex 4.6 x 45 mm, 5 µm Ultrasphere-ODS octadecylsilane precolumn and an Altex 4.6 x

250 mm, 5 μm Ultrasphere-ODS octadecylsilane analytical column maintained at 45°C.

Amino acid derivatives were detected using a Gilson model 121 fluorescence detector equipped with a 9 μL flow cell and filters for excitation at 305 and 395 nm and emission at 430 to 470 nm. Detector range and time constant settings were 0.02 relative fluorescence units and 0.5 s, respectively. Amino acids in tissue extracts were identified by comparison of their retention times to those of pure amino acid standards (Sigma Chemical Co.) and by coinjection of samples and the standards. Peak areas were determined using a Nelson Analytical (Cupertino, CA, U.S.A.) model 4416X chromatography data system. Amino acids were quantified using standard curves and yields of individual amino acids in extracts were calculated based on recoveries of the internal standard.

Plant survival and seedling height were expressed as a proportion relative to the control treatment and were calculated for each replicate according to the following equations: seedling height = (height - mean height of control)/mean height of control); plant survival = (plant survival - mean plant survival of control)/mean plant survival of control.

Analysis of variance was performed on DABA concentration of flatpea tissues and DABA means for different doses of gamma radiation were compared using Fisher's Least Significant Difference (LSD) multiple range test. A general linear models regression analysis was used to test the relationship between gamma radiation dose and seedling height and plant survival.

RESULTS

Although irradiation treatments did not reduce seed germination relative to the nonirradiated controls, percentage reduction in plant survival compared to the control treatment increased linearly with increasing gamma ray dosage (Fig. 2). LD_{50}, LD_{25} and LD_{10} values, calculated from the regression equation of plant survival on dosage, were 13.8, 11.0, and 9.4 kR, respectively. A corresponding reduction of seedling height with increasing irradiation was evident after a 3 wk growth period (Fig. 2). Reduction in vigor was no longer apparent when M_1 plants matured.

No clear-cut relationship between gamma radiation dose and DABA concentration was observed, although 12.5 kR gamma irradiation did significantly reduce mean DABA concentration compared to the nonirradiated control (Table 7). M_2 plants were found with as low as 6.6 mg g^{-1} dry wt. foliar DABA compared to a minimum value of 17.3 mg g^{-1} dry wt. foliar DABA in nonirradiated plants. Average free amino acid concentrations of mutagenized plants from the four treatment levels were generally not statistically different from control values (Table 8).

Abnormal M_2 phenotypes produced in response to gamma irradiation were rarely observed and occurred primarily in the form of viable chlorophyll mutations. Leaf variegation appeared as white on green longitudinal stripes, or "striata" mutants, according to the classification of Basu and Basu (1969). Mutants exhibiting abnormal leaf and flower morphology were also recovered among M_2 plants, although in lower frequencies than chlorophyll mutations. Asymmetric flower mutants were characterized by a missing wing petal and an extra standard petal; leaf mutants appeared as curled margins near the tips of young leaves. Of 212 total plants observed over all gamma irradiation treatments, frequencies of chlorophyll, curled leaf, and asymmetric flowers were 3.3%, 2.4%, and 0.94%, respectively. Numbers of morphological mutants were not statistically related to radiation dose. Flower petal color was stable in both natural and mutagenized plant populations.

DISCUSSION

Dose response curves for seedling traits conformed with the results of other researchers by exhibiting a linear trend over a narrow mutagen treatment range (Constantin et al., 1976; Sharma and Sharma, 1986; Sinhad and Godward, 1972). Seedling lethality and plant survival were dose dependent, whereas free amino acid concentrations were not consistently related to radiation treatment.

M_2 plants containing the lowest levels of DABA and GABA originated from seeds treated with 12.5 and 10.0 kR gamma radiation, respectively (Table 7). An individual M_2 plant with only 6.6 mg g^{-1} foliar DABA was recovered from plants derived from seed treated with 12.5 kR gamma radiation. Despite the large range of DABA concentrations for the nonirradiated control plants (17.3 to 61.3 mg g^{-1}) mean control values of DABA (38.2 mg g^{-1}) were consistent with other reports (Shen, 1987). Using similar analytical methods to those used in this study, Shen (1987) reported a mean foliar DABA concentration of 25.7 mg g^{-1} (range of 16.7 to 34.0 mg g^{-1}) for 24 Lathco flatpea plants averaged over eight plant age groups.

Even in genotypes with reduced foliar DABA following seed irradiation, concentrations of protein amino acids remained stable (data not shown), suggesting that selection for low-toxin flatpea is possible without severe disruption of protein metabolism. Whereas the CV of GABA was greater in radiation-treated M_2 plants than the nonirradiated control, the CV of DABA was reduced by irradiation treatments (Table 7). Random mutation generally increases the variance in crops that have previously been subjected to breeding and selection (Brock, 1965), but flatpea may respond differently because it has not been selected for reduced DABA content. For both DABA and GABA, the 12.5 kR gamma radiation treatment produced the highest CV values.

Although plants exhibiting asymmetric flowers offer potential for use as marker traits to identify crosses, it is not known if the flower characteristics will be stably inherited. In addition, not all of the flowers of the identified plants produced the asymmetric mutation.

Larger samples of the mutated populations of flatpea need to be characterized and tested in succeeding generations to establish the heritability of the genotypes with apparently reduced DABA. Establishment of basic mutagenesis parameters and recovery of presumptive low-toxin mutants remains encouraging for development of improved flatpea cultivars.

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Abbreviations

DABA (2,4-diaminobutyric acid); GABA (4-aminobutyric acid); ODAP (3-N-oxalyl-2,3-diaminopropionic acid); Hse (homoserine); Asp (aspartic acid); Ala (alanine); Val (valine); Ile (isoleucine); Leu (leucine); Glu (glutamic acid); Asn (asparagine); Ser (serine); Gln (glutamine); Arg (arginine).

Table 7. Mean, range and coefficient of variation of nonprotein amino acids in mature leaves of M_2 plants following gamma irradiation of Lathco flatpea seed.

Gamma radiation	Amino acid		
	Mean[a]	Range	CV
(μM)	-- mg g^{-1} dry wt. DABA --		%
0.0	38.2	17.3 - 61.3	43.5
10.0	29.3	24.4 - 37.7	15.1
12.5	20.2	6.6 - 29.5	40.9
15.0	44.3	30.3 - 60.5	27.8
17.5	39.0	21.2 - 58.4	34.1
LSD (0.05)	14.3		
	-- mg g^{-1} dry wt. GABA --		
0.0	45.7	30.9 - 57.0	22.4
10.0	31.4	7.6 - 54.9	54.5
12.5	62.0	37.7 - 140	63.9
15.0	47.9	26.0 - 63.0	26.6
17.5	53.2	22.2 - 83.0	37.0
LSD (0.05)	26.7		

[a]Means represent six plants.

Table 8. Free amino acid composition of M_2 plants following gamma irradiation of Lathco flatpea seed.

Amino acid	Gamma radiation (kR) 0	10.0	12.5	15.0	17.5	LSD[a] (0.05)
	------------- mg g^{-1} dry wt.				---------------	
Hse	9.6	6.8	8.6	9.5	9.7	NS
Asp	1.1	0.89	1.1	1.1	0.86	NS
Ala	0.22	0.19	0.29	0.25	0.28	NS
Val	0.20	0.09	0.22	0.16	0.24	NS
Ile	0.12	0.06	0.24	0.08	0.14	NS
Leu	0.19	0.10	0.31	0.15	0.19	0.18
Glu	0.23	0.27	0.51	0.25	0.23	NS
Asn	0.33	0.16	0.64	0.40	0.41	0.45
Ser	0.57	0.59	0.58	0.97	0.62	NS
Gln	0.37	0.22	0.37	0.33	0.34	NS
Arg	0.14	0.13	0.26	0.17	0.14	NS

NS not significant

[a]Linear and quadratic trends not significant for all amino acids; means represent six plants.

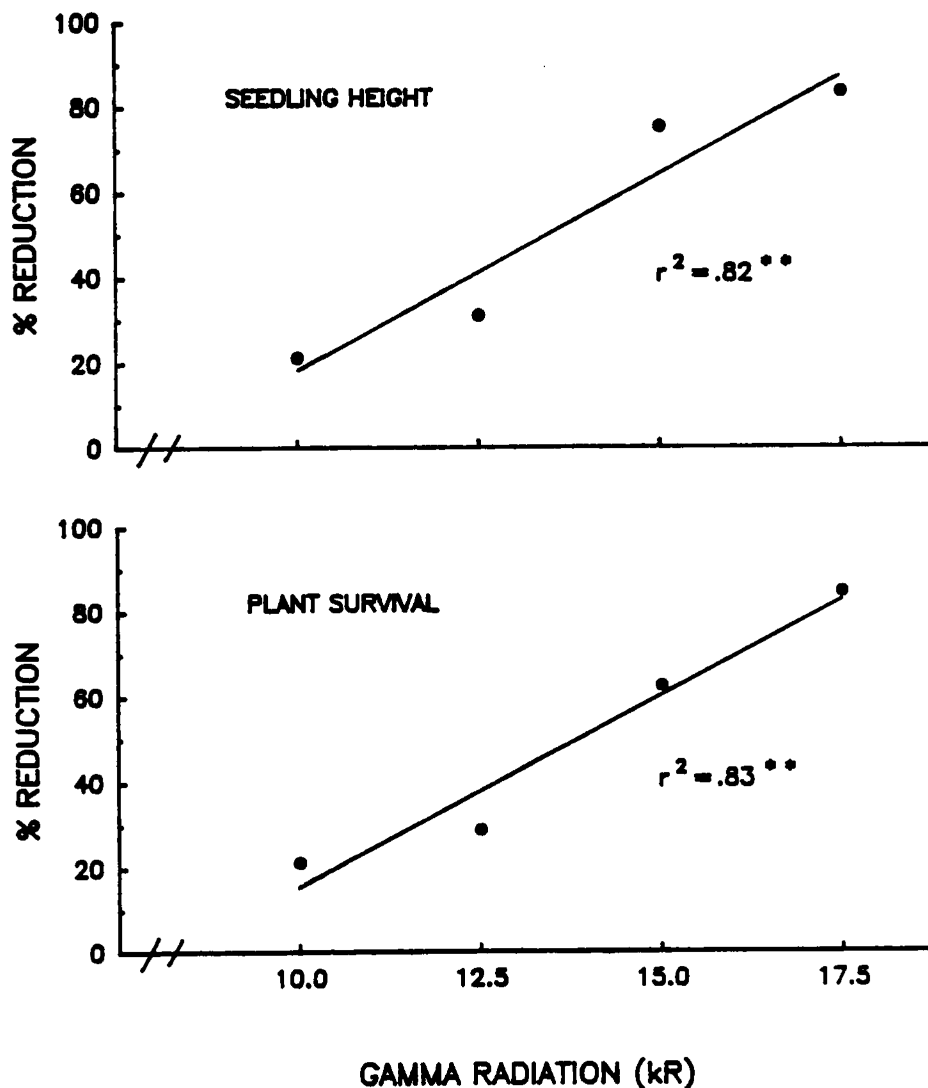

Fig. 2. Relationships between Lathco flatpea seed irradiation and seedling height and plant survival at 3 wk after planting (Fifty seeds were planted per treatment). Regression equations: seedling height = 0.0395 (x) -0.00506 (x^2) -0.0817 and plant survival = 8.96 (x) - 73.8 (**, F value significant at the 1% level). Seedling height = (height - mean height of control)/mean height of control); plant survival = (plant survival - mean plant survival of control)/mean plant survival of control.

Chapter V

FLATPEA POLLINATION METHODS AND GERMPLASM SCREENING FOR REDUCED 2,4-DIAMINOBUTYRIC ACID COMPOSITION

ABSTRACT

Flatpea (Lathyrus sylvestris L.) is a potentially valuable forage legume, but it contains high levels of 2,4-diaminobutyric acid (DABA), a compound that can have adverse effects on some animals, including rats and poultry. This study evaluated pollination methodology needed for cultivar improvement and characterized intercrossed progeny for DABA concentration. Pollen germination on an artificial medium using pollen collected during different stages of flower development was used to identify appropriate stages for flower emasculation. Pollen dehiscence occurred at the early-bud stage when petal color was first evident. Slightly more mature buds contained pollen with substantially higher in vitro germination than pollen from early-bud stage flowers. Lines that produced high numbers of seeds per pollination were identified from crosses among flatpea accessions. Cross-pollination resulted in significantly higher seed set than selfing. Pollination using caged honeybees was the most efficient method of hybridization, although the degree of inbreeding in bee-pollinated plants could not be determined. Intercrossed progeny produced by honeybee pollination contained a lower mean concentration of foliar DABA than the mean for flatpea accessions.

INTRODUCTION

Flatpea (Lathyrus sylvestris L) has several advantages as a forage and conservation legume for cool, temperate climates, including persistence on low-fertility soils and tolerance to environmental stresses. However, utilization of flatpea has been limited due to the possibility of detrimental effects on some animals consuming flatpea forage. Adverse effects of flatpea consumption have been observed in rats and

poultry (Huang et al., 1950; Miller, 1946) and Ressler (1961) has identified 2,4-diaminobutyric acid (DABA) as a neuroactive component of flatpea. Selection of flatpea for traits of interest has been hampered because of insufficient genetic variability. A single flatpea cultivar, 'Lathco', released in 1972 by the Soil Conservation Service, is commercially available.

Availability of flatpea cultivars with lower DABA content than the existing cultivar would enhance acceptance of flatpea by growers. A lack of information on breeding methods and limited diversity of flatpea germplasm have restricted progress in cultivar development. A recent report by Chaib et al., (1985) indicates that considerable variation for seed production and chromosome karyotype exists in flatpea populations. Their findings also suggested that flatpea is a facultatively allogomous species. Attempts to improve *Lathyrus* species through interspecific hybridization have generally been unsuccessful, despite a consistent chromosome number of 2n=14 among species (Senn, 1938; Davies, 1957). Other than these reports, little research has been conducted on flatpea cultivar improvement. In a species related to flatpea, *Lathyrus sativus* L., reduction of 3-N-oxalyl-2,3-diaminopropionic acid (ODAP, also referred to as β-oxalyl amino-L-alanine, or BOAA), a neuroactive component similar in structure to DABA, was achieved only by extensive screening of hybridized and mutagenized plants (Jeswari et al., 1970). *L. sativus* mutants containing no detectable ODAP have been recovered, but these plants exhibited extremely low seed set (Prasad and Das, 1980).

Objectives of the present study were to define conditions for effective cross-pollination of flatpea and to assemble accessions and intercrossed germplasm stocks as a base for selection of low-DABA genotypes.

MATERIALS AND METHODS

Pollen Germination

Determination of the appropriate flowering stage for effective cross-pollination to the exclusion of maternal self-pollination required characterization of the sequence of pollen shed in undisturbed flowers. Pollen viability was measured by collecting pollen at different stages of floral development and germinating the pollen on an artificial medium as described by Fell et al. (1983). Lathco flatpea plant used for pollen collection were grown in a controlled environment growth chamber programmed for an 18 h light/6 h dark cycle, 25° C, and 60% relative humidity. Seedlings were transplanted into 15.2-cm pots containing a peat moss and perlite potting mixture and timed release 17-6-12 fertilizer plus micronutrients (Sierra Chemical Co., Milpitas, CA, U.S.A.) without rhizobia inoculation. Plants were grown to maturity in the greenhouse with average temperatures of 24±2°C (day) and 19±2°C (night). Period of illumination was extended to 18 h using high pressure sodium lamps. Plants received weekly fertilizer additions of 2.2 g L^{-1} 20-20-20 liquid fertilizer. After 4 wk of growth, plants were transferred to 11.4 L capacity pots and were supported with woven wire (5.1 x 10.2 cm mesh) cylindrical trellises. Trellising had a secondary benefit of enhancing flowering due to increased light penetration into the canopy. Reduced flowering resulted if the light period was reduced to 16 h. A combination of cool white fluorescent and incandescent lamps produced an average illumination of 550 µmol m^{-2} s^{-1} at canopy height.

Flowers at six developmental stages were collected from established Lathco flatpea plants and placed in moist paper towels. Flowering stages were defined as follows: early-bud (no petal color evident), mid-bud (slight petal color in keel, no color in standard or wings), late-bud (moderate color, all petals), early-flower (standard petal partially separated along the suture), mid-flower (standard fully separated), and full flower (all petals fully-expanded). Fifteen flowers per develop-

mental stage were divided into three replications, each consisting of five flowers from an individual plant.

Pollen germination medium was prepared by heating a mixture of 10.0% (w/v) sucrose, 0.7% (w/v) phytagar, and 3.0 $\mu g\ g^{-1}$ boron (as boric acid) in distilled water (Fell et al., 1983). After vigorous shaking of the cooled medium to ensure aeration, a drop of partially solidified agar medium was placed on a microscope slide. A composite sample of pollen from the anthers of five flowers was collected in a gelatin capsule and applied to the drop of medium and a large cover slip was placed on top. Slides were incubated in the dark at 25° C for 12 h. Frequency of germinating pollen grains was determined microscopically (200X). Pollen tubes exceeding the diameter of the grain in length were considered viable in a sample of at least 250 grains per treatment.

Pollination Methods

Hand pollination techniques were modified from Combes and Delbos, University of Pau, France (personal communication). Accessions were provided by Dr. F. Bisby, University of Manchester, United Kingdom. In addition to the common U.S. cultivar, Lathco (Big Flats Plant Materials Center, Corning, NY, U.S.A., lot no. LASY81FR), the following British accession numbers were evaluated (country of origin indicated in parentheses): 780567, 780670, 780901 (U.S.S.R.); 780099, 780100 (East Germany); 780201 (United Kingdom). Five-month-old plants used for pollinations were grown in the greenhouse under conditions as described for pollen germination. Pot size and fertilization regime were as described for pollen collection. Pollinations were conducted during March 1987.

Flowers at early-bud stage were utilized as female parents. A separate set of five Lathco flatpea plants in each of three replications was used to test the following methods of hand pollination: concurrent emasculation and pollination, delayed pollination, cross-pollination without emasculation, and self-pollination by hand tripping. Delayed

pollinations were performed by application of pollen the morning following emasculation.

Overmature flowers and immature buds were removed from the inflorescence to reduce competition for nutrients. Buds were opened for removal of anthers by slitting along the fused margin of the standard petal with a scalpel. Separated petals were folded back and held lightly between the thumb and forefinger. Anthers were removed using 11.4-cm microforceps sterilized in ethanol. A small section (approximately 2 x 5 cm) of fine sandpaper was used to abrade the stigmatic surface. Pollen from a single flower of the male parent was transferred by pressing on the keel petal of mature flowers and brushing the extruded anthers directly onto the abraded stigma of the flower. To prevent desiccation of the pollinated flowers, petals were replaced to their original positions and secured with a strip (approximately 3 cm^2) of transparent tape.

Compatibility relationships were examined following hand-pollinated crosses among seven flatpea accessions in all possible combinations, including reciprocals. Three replications of each accession were arranged on greenhouse benches in a randomized complete block design with one plant per replication. An average of 42 delayed pollinations per replicate was performed for each of the 49 crosses in the diallel population. Mature seeds were harvested 6 to 7 wk following pollination. Numbers of pollinations, pods (containing at least one seed per pod), and total seeds per cross were recorded. Seeds per pollination, pods per pollination, and seeds per pod were calculated from these values. Self-pollination for the diallel population involved emasculation as previously described for evaluation of pollination methods followed by application of pollen from another flower of the same plant.

A standard five-frame nucleus honeybee hive enclosed in a 1.9 m^2 cage covered with plastic screen was used for open-pollinated intercrosses. The cage was placed in a walk-in growth chamber maintained at 22° C and programmed for a 16 h day/8 h night cycle. Honeybees were given access to a supplemental 50% (w/v) sucrose solution. The hive was located in the center of the cage and six trellised flatpea plants in full bloom were placed near the cage perimeter and rotated daily. In-

dividual plants that were placed in the bee cage had been selected based on indications of high seed set after hand pollination. Initial numbers of flowers present on each plant were recorded.

2,4-Diaminobutyric Acid (DABA) Analysis

Plants from random samples of bulked seed produced by bee-pollinated intercrosses were grown in the walk-in growth chamber under identical conditions as described for plants used for pollen germination testing. A total of 24 3-month-old F_1 plants and six plants of each accession were randomly selected and screened for DABA concentration. Analytical procedures have been described in detail by Foster et al. (1987). Fully expanded mature leaves were harvested and crushed in a mortar containing liquid nitrogen.

For analysis of free amino acids, 0.2 to 0.4 g of lyophilized tissue was extracted for 90 min using 90 ml of 50% (v/v) aqueous ethanol in 30 mm i.d. Soxhlet extraction tubes. Two ml of 15 mM S-(4-pyridylethyl)-DL-penicillamine (Pierce Chemical Co.. Rockford, IL, U.S.A.) was added as an internal standard. Sample extracts were concentrated under nitrogen at 40°C; the resulting residue was resuspended in 10 ml of extraction medium. A 2.5 ml aliquot of this suspension was centrifuged at 3,000 g for 10 min. The supernatant was brought to a total volume of 7.5 ml with extraction medium; 0.25 ml of this preparation was loaded onto a Sep-Pak C_{18} column (Waters Associates, Milford, MA, U.S.A.) and eluted successively with 0.5 ml of water and 1.0 ml of methanol. Combined eluates were adjusted to a final volume of 2.0 ml with water.

Free amino acids in extracts were derivatized with o-phthalaldehyde (OPA, Pierce Chemical Co.) prepared by dissolving 50 mg of OPA in 1 ml of HPLC-grade methanol, adding 50 µL of 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and bringing the solution to a final volume of 10 ml with 0.40 M sodium borate-KOH, pH 9.5 containing 0.1% (v/v) Brij 35 (polyoxyethylene lauryl ether, Fisher Scientific, Pittsburgh, PA, U.S.A.). Freshly prepared OPA stock solution was stored overnight at 0 to 5°C before use and was used for 2 d. A 0.1 ml aliquot

of the solution was mixed with 0.02 ml of extract 90 s before injection onto the HPLC column. Derivatized samples were analyzed using a Beckman model 344 binary gradient HPLC system equipped with an Altex 4.6 x 45 mm, 5 µm Ultrasphere-ODS octadecylsilane precolumn and an Altex 4.6 x 250 mm, 5 µm Ultrasphere-ODS octadecylsilane analytical column maintained at 45°C.

Amino acid derivatives were detected using a Gilson model 121 fluorescence detector equipped with a 9 µL flow cell and filters for excitation at 305 and 395 nm and emission at 430 to 470 nm. Detector range and time constant settings were 0.02 relative fluorescence units and 0.5 s, respectively. Amino acids in tissue extracts were identified by comparison of their retention times to those of pure amino acid standards (Sigma Chemical Co.) and by coinjection of samples and the standards. Peak areas were determined using a Nelson Analytical (Cupertino, CA, U.S.A.) model 4416X chromatography data system. Amino acids were quantified using standard curves and yields of individual amino acids in extracts were calculated based on recoveries of the internal standard.

Statistical Analysis

Analysis of variance was performed on pod and seed set per pollination and DABA concentration of flatpea germplasm. A SAS general linear models (GLM) analysis was used for analysis of variance of pollen germination frequency due to unbalanced data. Means for different methods of pollination, individual crosses and DABA means for different tissues were compared using Fisher's Least Significant Difference (LSD) multiple range test.

RESULTS

A combination of in vitro pollen germination data (Fig. 3) and microscopic examination of pollen dehiscence showed that early-bud stage flowers constitute the optimum flower stage for hand cross-pollination of flatpea. Mid-bud stage flowers exhibited an average of 17.4% pollen germination. Although some pollen dehiscence was observed in early-bud stage flowers, only 3.3% of the released pollen germinated on the artificial medium (Fig. 3). Thus, utilization of mid-bud stage flowers as female parents would involve some risk of self-pollination, whereas early-bud stage flowers would not contain significant levels of viable pollen. Mid or late stage flowers produced the highest levels of pollen germination and would be satisfactory sources of male parent pollen.

Delayed application of foreign pollen until the morning following emasculation effectively doubled both pod set and seeds per pollination compared to concurrent emasculation and pollination (Table 9). Pods per pollination increased when pollen was applied without emasculation, indicating that some damage due to manipulation occurred during removal of anthers. Of 448 undisturbed flowers on plants that were protected from insects in a greenhouse, only four seed-containing pods were found, indicating very low levels of self-fertilization in the absence of tripping. However, self-pollination by hand tripping produced nearly as many seeds as crossing without emasculation. Seed set increase was greatest after honeybee pollination, with most of the increase due to a greater number of seeds per pod (Table 9). The number of pods produced using honeybees was equivalent to that produced using hand pollination without emasculation and significantly higher than that obtained with emasculation.

Mean seed and pod sets per pollination for the accessions used in the diallel crosses are shown in Tables 10 and 11, respectively. When used as the female parent, accession 780567 was clearly superior to the other entries in seeds produced per pollination (Table 10). No differences were observed among accessions serving as the male parent. Significantly higher seed set was observed in cross- compared to self-

pollinated flowers. Self-pollinated flowers produced 0.40 seeds per pollination, indicating that flatpea is at least partially self-compatible.

Number of seeds per pod was generally constant for hand crosses regardless of the individual crosses, but increased from 0.40 to 0.93 for cross-pollination compared to selfing. Pod set after hand-pollination was 0.40 and 0.18% for cross- and self-pollination, respectively. Both self- and cross-pollination involved flower emasculation for these comparisons within the diallel flatpea population. Pod formation in the absence of pollination was observed in most flowers, although seeds failed to develop in pods.

Mean foliar DABA concentration of plants selected at random from the population of intercrossed progeny was 30.6 mg g^{-1} compared to a mean of 36.8 mg g^{-1} for the pooled flatpea accessions (Table 12). Intercrossed progeny showed a lower range and coefficient of variation in foliar DABA concentration than accessions. Lathco contained an average of 42.2 mg g^{-1} foliar DABA, compared to an average of 36.8 mg g^{-1} in the accessions. Of the accessions screened, 780201 contained the lowest mean DABA concentration (24.0 mg g^{-1}), although both accession 780567 and 780901 and intercrossed progeny contained individual genotypes with foliar DABA similar to the lowest DABA-containing genotype in 780201.

DISCUSSION

Observations of partial self-incompatibility in flatpea and the shedding of viable pollen at mid-bud flower stage (Fig. 3) suggest that critical crossing studies in flatpea should utilize young flower buds to avoid accidental self-pollination. At present, viable marker genes have not yet been identified to enable verification of flatpea hybrids. Elimination of possible self-pollination through mechanical emasculation is therefore essential to ensure true crosses.

Higher cross-fertility of flatpea and the apparent requirement for tripping to produce fertilization suggest that natural populations of flatpea are predominantly cross-pollinating. However, estimates of

natural crossing using hand pollination with and without emasculation may not be entirely valid due to the absence of competition between self- and cross-pollen that occurs in the field (Scheetz, 1972). A facultative outcrossing mode of pollination is in agreement with the findings of Combes and Delbos (1985, personal communication) and has been found in other forage legumes (Armstrong and White, 1935; Cope, 1966; Scheetz, 1972). Natural cross-fertilization in some legumes has been attributed to coincidental arrival of foreign pollen with the tripping insect and rupturing of the stigmatic surface during the tripping process (Armstrong and White, 1935; Rowlands, 1958). The present study does not provide direct information as to whether flatpea is similar to alfalfa in producing greater frequencies of cross than self seed even when both self and cross pollen are present on the stigma (Hanson et al., 1964). Percentage pod set in alfalfa after manual tripping ranged from 9 to 56%, depending on the use of either high or low pod-setting genotypes (Armstrong and White, 1935).

Effectiveness of honeybees in flatpea pollination is likely due to frequent bee visitations that eventually deliver pollen during periods of stigmatic receptivity, coupled with the effects of flower tripping. Temporal separation of stigmatic receptivity and pollen dehiscence in flatpea is supported by the notable increase in seed set when pollen application in hand crosses was delayed (Table 9). Intercrossing of flatpea with honeybee pollinators was the most labor-efficient means of crossing evaluated, although a mixture of self and cross seed was likely produced. Hanson et al. (1964) estimated that more than half of the seed in bee-pollinated crosses of alfalfa were self-pollinated. Crossing effectiveness using honeybees has been shown to vary widely depending on plant spacing, pollinator attraction, ease of tripping, and environmental factors (Kehr, 1973). Additionally, nonrandom pollination can occur due to honeybee foraging patterns that show preference for particular clones (Boren et al., 1962). Despite these sources of variation influencing intercrossed progeny, foliar DABA concentrations of pooled accessions displayed the greatest range and CV. Greater variability in flatpea accessions than in intercrossed progeny may be

related to the relatively lower sample size of intercrossed progeny and to the lack of previous selection for low DABA in intercrossed plants.

DABA content of accessions did not indicate an association with differences in combining ability for seed set. Nonprotein amino acids, including DABA, can form effective hybridization barriers between Lathyrus species (Simola, 1967; Bell and Fowden, 1964). Simola (1967) demonstrated that in vitro germination of pollen and growth of pollen tubes was inhibited by DABA. Further studies designed to intercross accessions that have been selected for low toxin content would likely be effective in reducing DABA content of flatpea.

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Table 9. Seed and pod set per pollination or available flower and seeds per pod after five methods of pollination within Lathco flatpea.

Method of pollination	Seeds per pollination	Pods per pollination	Seeds per pod
	---------- Mean no.[a] ----------		
Concurrent emasculation and pollination	0.52	0.21	2.6
Delayed pollination	1.18	0.45	2.6
Cross-pollination without emasculation	1.75	0.75	2.3
Self-pollination by hand tripping	1.44	0.64	2.3
	Seeds per available flower	Pods per available flower	Seeds per pod
	---------- mean no. ----------		no. pod^{-1}
Caged honeybees	2.42	0.70	4.0
LSD (0.05)	0.66	0.22	1.5

[a]Means represent four replications (average of 19 pollinations per replicate)

Table 10. Seed set per pollination in a complete diallel population composed of seven flatpea accessions.

	Female parent (Accession no.)							
Male parent	780567	780099	Lathco	780100	780901	780670	780201	LSD (0.05)
(Accession no.)	Mean no. seeds per pollination[a]							
780567	0.71	1.4	1.8	1.6	2.4	1.8	1.1	0.89
780099	1.6	0.41	0.99	0.85	0.53	0.91	0.64	NS
Lathco	1.3	1.0	0.18	0.77	0.72	0.70	0.94	NS
780100	0.86	0.87	1.4	0.51	0.58	1.0	1.0	NS
780901	0.93	1.1	0.27	0.82	0.25	0.83	0.54	NS
780670	0.64	0.83	0.42	0.66	0.51	0.49	0.87	NS
780201	1.0	0.64	0.57	0.50	0.46	0.87	0.27	NS
LSD (0.05)	NS	NS	0.74	NS	0.74	NS	NS	
Self	0.40							
Cross	0.93							
Self vs Cross	**							

** significant contrast at 1 % level of probability
NS not significant
[a]Means represent three replications (average of 42 pollinations per cross).

Table 11. Pod set per pollination in a complete diallel population composed of seven flatpea accessions.

Male parent	Female parent (Accession no.)							
	780567	780099	Lathco	780100	780901	780670	780201	LSD (0.05)
(Accession no.)	Mean no. pods per pollination[a]							
780567	0.23	0.50	0.64	0.48	0.91	0.76	0.52	0.32
780099	0.67	0.16	0.45	0.35	0.26	0.34	0.35	NS
Lathco	0.60	0.30	0.12	0.33	0.39	0.28	0.56	NS
780100	0.34	0.34	0.59	0.21	0.33	0.38	0.55	NS
780901	0.40	0.49	0.18	0.33	0.12	0.38	0.23	NS
780670	0.31	0.31	0.22	0.30	0.27	0.24	0.41	NS
780201	0.45	0.29	0.22	0.26	0.23	0.34	0.16	NS
LSD (0.05)	NS	NS	0.33	NS	0.35	NS	NS	
Self	0.18							
Cross	0.40							
Self vs Cross	**							

** significant contrast at 1% level of probability

NS not significant

[a]Means are an average of three replications (average of 42 pollinations per cross).

Table 12. Mean, range, and coefficient of variation (CV) of foliar 2,4-diaminobutyric acid (DABA) in flatpea accessions and intercrossed progeny. Mean seed set for each accession are shown for comparison.

Accession	Seed set	DABA Mean	DABA Range	DABA CV
	(Seeds per pollination)	----- mg g^{-1} dry wt. -----		%
780099	0.85	47.7[a]	29.0 - 71.7	32.6
780100	0.89	45.4	27.8 - 61.3	27.6
780670	0.63	43.3	24.0 - 55.1	27.4
Lathco	0.80	42.2	24.7 - 53.6	24.5
780567	1.56	28.9	9.0 - 41.5	47.0
780901	0.70	26.1	12.9 - 32.2	26.2
780201	0.61	24.0	11.9 - 38.2	39.7
pooled accessions		36.8	9.0 - 71.7	32.0
Intercrossed progeny		30.6[b]	11.0 - 49.8	23.9

[a]Means for accessions represent six plants.
[b]Means for intercrossed progeny represent six replications (four plants per replicate).

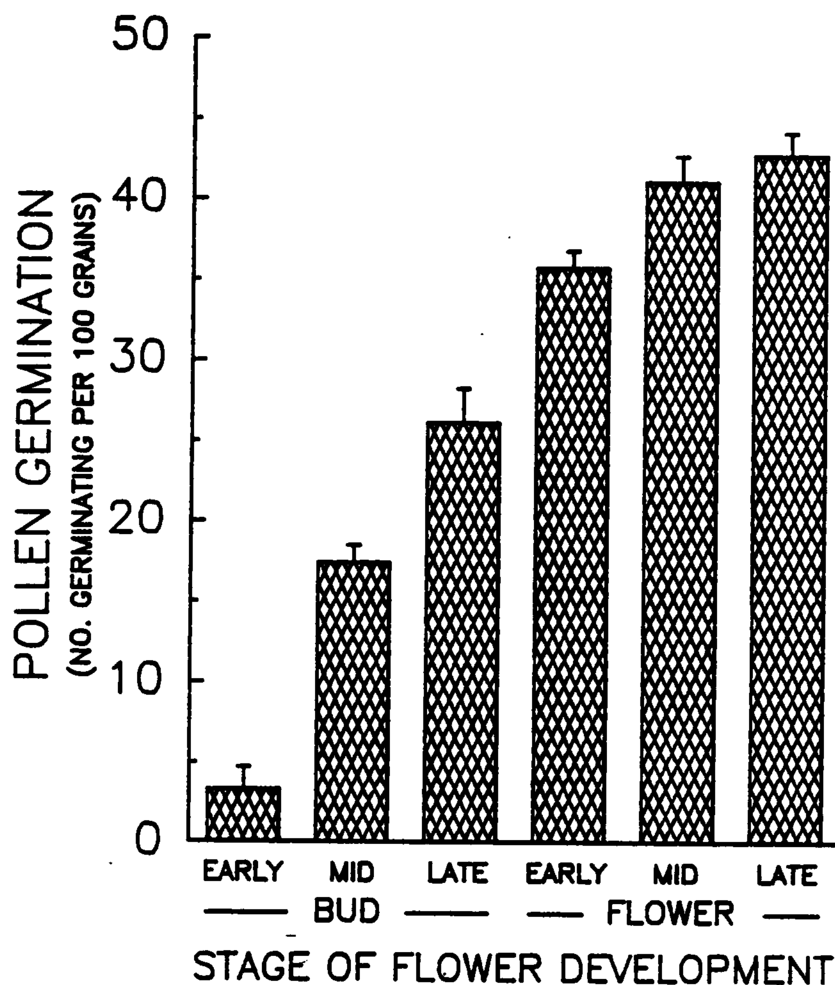

Fig. 3. Frequency (no. germinating per 100 pollen grains) of in vitro germination for flatpea pollen collected from flowers at different stages of development. Error bars indicate standard error of means within a flower stage. Pollen germination means are based on a minimum of 250 pollen grains per developmental stage.

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