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HYBRIDIZATION STUDIES USING AUSTRALIAN C_4 ATRIPLEX SPECIES AND THE C_3 SPECIES A. PROSTRATA

L. M. BIELIG AND P. F. BROWNELL

Department of Botany, James Cook University of North Queensland, Townsville, Queensland 4811, Australia

To elucidate the mechanisms of inheritance of the C_4 photosynthetic pathway, C_4 species of *Atriplex*, endemic to Australia, were crossed as female parents with the C_3 species *A. prostrata*, which is regarded as a relatively recent introduction to Australia. Seed set ranged from 0.9% to 73.1%, depending on the particular female parent involved and the duration of pollen application. Of 102 seeds in embryo culture, only two F_1 individuals survived to maturity. Their morphology, leaf anatomy, and fruit structure showed hybrid characteristics. Pollen grain viability was only 7.8% and 2.0%, respectively, in the two hybrids compared with pollen grain viability in excess of 95% in each parent. Of 1,160 fruits examined from the F_1 plants, only 12 contained full seeds, while 67 contained endosperm-deficient seeds. These data indicate a very distant relationship between these Australian C_4 species and *A. prostrata*. In order to investigate the genetic control of C_4 photosynthesis, it may be necessary to generate interspecific $C_4 \times C_3$ hybrids via protoplast fusion.

Introduction

Plant breeders interested in incorporating at least some of the characteristics of C_4 photosynthesis into agronomically important plants require an understanding of the genetic control of C_4 photosynthesis. For this reason, attention has focused on genetic studies within genera which include both C_4 and C_3 species: *Atriplex* is one such genus. The aim of the research reported here was to elucidate the genetic control of the C_4 pathway in *Atriplex*, by hybridization of C_4 with C_3 plants. It was anticipated that analysis of the F_1 and F_2 progeny with regard to segregation for characteristics of C_4 photosynthesis such as leaf anatomy and requirement for sodium, would clarify the patterns of inheritance of C_4 photosynthesis.

BJORKMAN et al. (1970), NOBS et al. (1970), and NOBS (1976) worked extensively on the production and analysis of hybrids between the C_4 species, *A. rosea* L. and the C_3 species, *A. triangularis* Willd. However, inadequate F_2 populations were obtained to analyze the inheritance of the C_4 pathway (BJORKMAN 1976). *Atriplex triangularis* Willd. which is now regarded as an illegitimate name for *A. prostrata* Boucher ex DC. (BASSETT et al. 1983) is a native of North America, Europe, and Asia, while *A. rosea* is of Eurasian origin. We determined whether crosses between C_4 *Atriplex* species endemic to Australia and *A. prostrata* yielded sufficiently large populations to elucidate the mechanisms controlling inheritance of C_4 photosynthesis.

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Address for correspondence and reprints: Dr. L. M. BIELIG, Department of Botany, James Cook University of North Queensland, Townsville, Queensland 4811, Australia.

Material and methods

PLANTS

The C_3 species, *Atriplex prostrata* Boucher ex DC., was crossed as male parent with six different C_4 accessions. Seeds of the C_4 parents were supplied by Dr. M. NOBS, Carnegie Institution of Washington, Dr. B. G. THOMSON, Department of Primary Industries, Alice Springs, and Mr. W. E. MULHAM, CSIRO, Deniliquin. New accessions received at the James Cook University were assigned a T (Townsville) accession number to differentiate each seed source. This was necessary because the taxonomy of *Atriplex* was confused. Although this has been largely rectified (WILSON 1984), there still exist areas of taxonomic uncertainty. When necessary, specimens were sent to Dr. PAUL WILSON, W. A. Herbarium, Perth, for positive identification.

The six C_4 accessions used and their accession numbers were: *A. limbata* Benth. (T22); *A. eardleyae* Aellen (T21); *A. lindleyi* subsp. *inflata* (F. Muell.) Paul G. Wilson (T24); *A. lindleyi* Moq. subsp. *lindleyi* (T3); *A. lindleyi* subsp. *conduplicata* (F. Muell.) Paul G. Wilson (T4), and a natural hybrid of T3 × T4 (T26). Seed of *A. prostrata* Boucher ex DC. (= *A. hastata* auct.) (= *A. triangularis* Willdenow) was already held at James Cook University, so no accession number was assigned. The identity of this material was confirmed by Dr. P. M. TASCHEREAU, Dalhousie University, Halifax.

Plants were grown in vermiculite in the glasshouse and provided with a mineral solution: 4,000 μM $Ca(NO_3)_2$; 6,000 μM KNO_3; 1,000 μM KH_2PO_4; 1,000 μM $MgSO_4$; 1,000 μM $(NH_4)_2HPO_4$; 44.7 μM ferric ammonium ethylenetetraacetate; 23.1 μM H_3BO_3; 4.5 μM $MnCl_2$; 0.4 μM $ZnSO_4$; 0.15 μM $CuSO_4$, 0.05 μM Na_2MoO_4.

Crosses

Atriplex prostrata was always used as male parent because in this species pistillate and staminate flowers are small and too closely associated to permit effective emasculation. The C_4 species chosen as female parents were selected from a range of *Atriplex* species because their floral morphology was such that the female flowers could be isolated. In these species, male flowers occur in terminal glomerules and female flowers in distinct axillary clusters. All terminal flowers were removed prior to anthesis, and the resulting female branches were bagged. Plants were checked daily and any staminate buds removed. Pollen from *A. prostrata* was applied to female flowers for as long as the stigmas appeared receptive. As new female flowers were continually maturing on any one plant, pollen was applied for between 1–5 wk, depending on the availability of pollen. Branches were rebagged after each pollen application. After several weeks, when fruiting bracts had ripened, fruits were examined to determine seed formation.

To confirm that the pollination technique was an appropriate means of ensuring maximum seed set, *A. eardleyae* flowers were emasculated, and pollen from other *A. eardleyae* was applied for as long as the stigmas were receptive. Seven selfings were carried out.

Other emasculated plants were used to test for the occurrence of apomixis. "Female isolates" of four accessions, T21, T22, T24, and T26 were produced by the removal of male flowers, prior to anthesis. The female isolates were kept under bags to prevent pollination. When the fruiting bracts ripened, the fruits were examined for seeds.

Embryo culture

To obtain viable F_1 seedlings, embryos were excised under aseptic conditions and grown on 0.8% agar containing one-fifth strength mineral solution (described above) and 6 mM sucrose. Embryos excised from seed obtained by selfing each parent served as controls. After 7–10 d, the developing seedlings were transferred to the glasshouse and planted in vermiculite with their roots still embedded in the solid agar medium.

Analysis of F_1 plants

Whole-plant and floral morphology as well as leaf size, shape, and color of F_1 plants were analyzed. Leaf structure was examined by light microscopy, using leaf tissue which had been fixed in 2.5% glutaraldehyde in 0.025 M phosphate buffer, embedded in Spurr epoxy resin, cut 1–2 μm thick and stained with 1.0% toluidine blue in 1% borax.

Inflorescences were fixed in Carnoy's solution (6:3:1) for 48 h and stored refrigerated in 70% ethanol. Squashes, made in acetocarmine, were examined using phase contrast optics. Fresh pollen grains were stained in acid fuchsin in lactophenol, to determine their viability. Viable pollen grains stained deep pink while nonviable grains remained yellow. Morphology of mature fruits was examined and seed set was ascertained by dissection of the ripened fruits.

Results

The selfings of *Atriplex eardleyae* to assess the efficacy of the crossing technique yielded 49 seeds in 69 female flowers pollinated (71% seed set). Crosses made when pollen was plentiful yielded 48 seeds in 52 flowers (92.3% seed set), while crosses made when the pollen supply was poor resulted in only one seed in 17 flowers (6% seed set). These data indicate that, provided adequate pollen is available, poor seed set in crosses is attributable to incompatibility between the parental species rather than inappropriate pollination technique.

In the test for apomictic seed set, 331 female flowers were isolated. No seeds formed on any of the female isolates. Dissection of the mature fruiting bracts showed the dried remains of ovaries and styles but no seeds. The absence of apomictic seed formation indicated that seeds formed on pollinated female parents were hybrid.

Crosses

All parents were $2n = 18$ chromosomes; seed set in crosses between six C_4 accessions of *Atriplex* and the C_3 species *A. prostrata* varied, depending on the female parent used (table 1). In *A. limbata* × *A. prostrata* only four of the 468 pollinated female flowers yielded seed (0.9%). By contrast, crosses with *A. eardleyae* yielded 30 seeds in 52 flowers pollinated (58%).

The remaining four accessions used as female parents belong to the Spongiocarpus section. Seed set ranged from 2.0% in one cross involving *A. lindleyi* (T3) to 73% in the cross with accession T26 (table 1). The differential seed set in the crosses involving T3 and T4 is attributed to the variation in the availability of pollen. When pollen was applied over 5 wk, seed set was in the order of 60%, whereas only 2.0% seed set was observed when the pollination period was restricted to 3 d.

All seeds were extremely small, less than one-quarter the size of selfed seed obtained from each parental species. Microscopic examination indicated that the seeds contained a small embryo and an extremely shrivelled endosperm. Thus, the embryos were excised and cultured in vitro. The only F_1 seeds not cultured in vitro were the first four obtained from *A. limbata* × *A. prostrata*. Three of the four seeds germinated readily, but the seedlings remained viable for only 5–6 d.

TABLE 1

SEED SET IN CROSSES BETWEEN SIX C_4 ACCESSIONS OF ATRIPLEX AND THE C_3 SPECIES ATRIPLEX PROSTRATA

C_4 species (female parent)	Accession number	Seed set/ number flowers pollinated	% seed set	Number of seeds embryo cultured
Section Semibaccatae:				
A. limbata	T22	4/468	.9	0
A. eardleyae	T21	30/52	57.7	14
Section Spongiocarpus:				
A. lindleyi:				
subsp. *inflata*	T24	13/21	61.9	13
subsp. *lindleyi*	T3	31/52[a]	59.6[a]	30
		1/49[b]	2.0[b]	
subsp. *conduplicata*	T4	37/64[a]	57.8[a]	31
		5/18[c]	27.8[c]	
T3 × T4 hybrid	T26	19/26	73.1	14

[a] Pollen applied daily for 5 wk.
[b] Pollen applied for only 3 d.
[c] Pollen applied daily for 12 d.

EMBRYO CULTURE

Of the 102 embryos aseptically excised and cultured in vitro only two survived to maturity; one involved *A. lindleyi* subsp. *inflata* and one subsp. *lindleyi* as female parent (table 1). By contrast, all 60 of the control embryos, excised from seeds of the appropriate parental accessions, developed into viable seedlings.

F_1 PLANTS

Pollen mother cells of all parental accessions yielded clear squash preparations, showing nine bivalents at metaphase I. From the two F_1 individuals, however, no countable squashes were obtained. Bivalents could not be clearly distinguished. Pollen grain viability in the two F_1 individuals was only 7.8% and 2.0%, respectively, while the parental accessions showed pollen viability of 95.2% and 98.7% (table 2).

The *A. lindleyi* subsp. *inflata* × *A. prostrata* hybrid was intermediate between the C_4 and the C_3 parent with regard to several characteristics, including height, floral morphology, and leaf color (table 3). As indicated by leaf tracings, leaf morphology of the F_1 was distinct from either parent as the leaves were sessile with entire margins (fig. 1). This and the presence of some leaf vesicles on the epidermis of the F_1, but far fewer than in the C_4 parent, were indicative of the hybridity of the F_1. The leaf anatomy substantiated that: the F_1 was quite distinct from either parent (fig. 2). The C_4 parent possessed Kranz anatomy with a conspicuous bundle sheath consisting of thick-walled cells surrounding the vascular tissue. A discrete layer of mesophyll cells enclosed the bundle sheath; there were one to two layers of spongy mesophyll cells between the palisade mesophyll and the epidermis. The leaf of the C_3 parent lacked a bundle sheath: the thin-walled spongy mesophyll cells which lay adjacent to the vascular tissue were not differentiated from spongy mesophyll cells found elsewhere in the leaf. The F_1 was clearly intermediate in leaf structure; the vascular bundle was sur-

TABLE 2

STAINABILITY OF POLLEN GRAINS IN PARENTS AND F_1 HYBRIDS FROM THE CROSS OF ACCESSIONS T3 AND T24 × ATRIPLEX PROSTRATA

Source of pollen	Ratio of stainable (pink) : nonstainable (yellow) pollen grains	% viability
A. lindleyi subsp. *lindleyi* (T3)	3353 : 103	97.0
F_1 of T3 × *A. prostrata*	289 : 3378	7.8
A. prostrata	3175 : 161	95.2
A. lindleyi subsp. *inflata* (T24)	5675 : 75	98.7
F_1 of T24 × *A. prostrata*	93 : 4557	2.0

TABLE 3

WHOLE-PLANT MORPHOLOGY, FLORAL MORPHOLOGY, LEAF SIZE AND COLOR IN ATRIPLEX LINDLEYI SUBSP. INFLATA, A. PROSTRATA, AND THE F_1 BETWEEN THEM

Character	*A. lindleyi* subsp. *inflata* (C_4)	*A. prostrata* (C_3)	F_1
Whole plant morphology	Erect, 35 cm tall; intricately branched	Variable, prostrate to erect plants 20–38 cm tall; diffusely branched	Semi-prostrate plant, 17 cm tall, branched profusely
Floral morphology	Female flowers in axillary clusters along full extent of stem; male flowers terminal in short glomerules 3–5 mm long	Terminal inflorescences 15–30 mm long contain male and female flowers; axils of lower branches bear 3–5 mm long inflorescences with numerous female and some male flowers	Axillary and terminal inflorescences very short (2–3 mm long) contain both male and female flowers
Leaf color, epidermal morphology	Leaves gray in color: the upper and lower epidermis covered with a layer of densely packed bladder-like vesicles	Leaves light green; no vesicles are present	Leaves light green with a distinct grayish tinge due to the presence of some vesicles but far fewer than in the C_4 parent
Leaf length (including petiole); shape	Leaves 15–25 mm long; obovate with dentate margins; leaf tapers into a narrowly cuneate petiole about 2 mm long	Leaves 25–32 mm long subtended by a petiole 5–9 mm long; triangular in shape with irregularly dentate margins	Leaves sessile, 12–20 mm long; margins entire

rounded by a readily discernible ring of bundle sheath cells. However, this sheath was much less developed than that of the C_4 parent; the thick walls that characterize the bundle sheath in *A. lindleyi* subsp. *inflata* were lacking in the F_1.

Fruit shape and structure are an important characteristic in differentiating *Atriplex* species. The fruits borne by the F_1 plant differed from those of each parent (fig. 3). Fruiting bracteoles of *A. lindleyi* subsp. *inflata* are spongy, subglobose, and pale brown, whereas the bracteoles of *A. prostrata* are thin and papery, vary from entire to dentate, are triangular to hastate, and are black at maturity. The bracteoles of the F_1 became black at maturity as did those of *A. prostrata* and tended toward the globose shape of subsp. *inflata* fruits. The fruits of the F_1 were not spongy and bore sculpturing and spines which were not present in either parent.

The low frequency of F_2 seed set by the F_1 plants was in accordance with the low pollen viability (table 2). In 460 fruits examined from the F_1 of *A. lindleyi* subsp. *inflata* × *A. prostrata*, only five full seeds and 32 endosperm-deficient seeds were formed. Similarly, 700 fruits from the second F_1 individual (subsp. *lindleyi* × *prostrata*) yielded only seven full seeds and 35 incomplete seeds.

Discussion

This is the first report of hybridization involving C_4 species of *Atriplex* endemic to Australia and a C_3 *Atriplex* species. The first artificial hybridization of $C_4 \times C_3$ *Atriplex* species involved two species naturalized in North America, *A. rosea* × *A. triangularis* (= *A. prostrata*) (BJORKMAN et al. 1970). The resulting F_1 seeds germinated readily and produced vigorous F_1 seedlings (NOBS et al. 1970). This was not our experience: the extremely poor viability of the F_1 seedlings and the very low

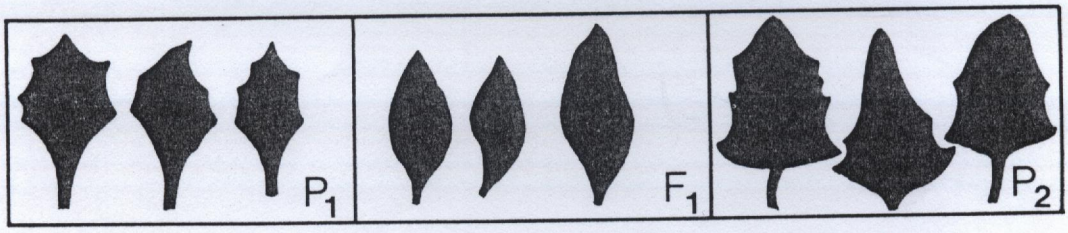

FIG. 1.—Leaf shape and size of *Atriplex lindleyi* subsp. *inflata* (P_1), *A. prostrata* (P_2), and their F_1 hybrid

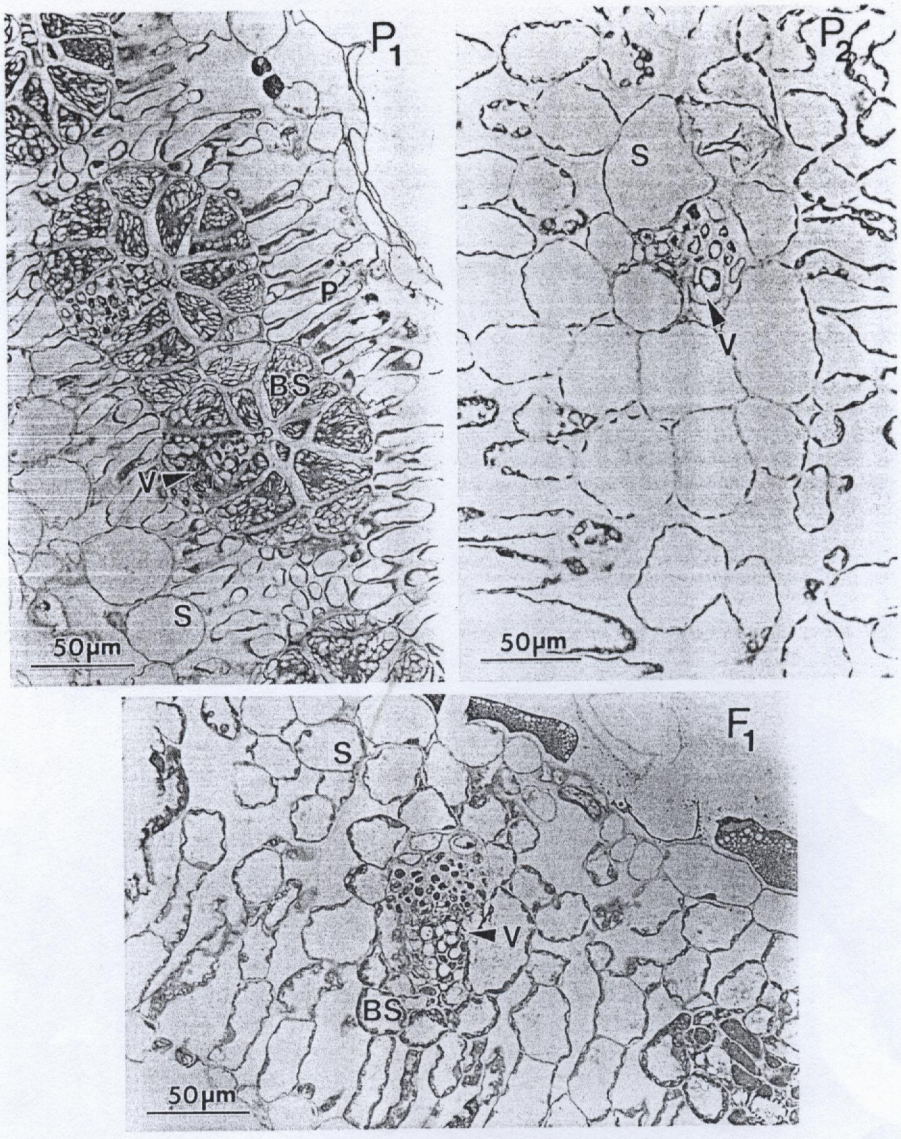

FIG. 2.—Light micrograph of cross sections of leaves of *Atriplex lindleyi* subsp. *inflata* (P_1), *A. prostrata* (P_2), and their F_1 hybrid. Vascular tissue (*V*) is enclosed in a bundle sheath (*BS*) in the C_4 parent and in the F_1, whereas in the C_3 parent undifferentiated spongy mesophyll (*S*) is associated with the vascular tissue. The bundle sheath in the C_4 parent is surrounded by a discrete layer of palisade mesophyll (*P*) cells.

frequency of F_2 seed set indicate that a strong compatibility barrier exists between the Australian C_4 species used and *A. prostrata*. The latter, a native of Europe, Asia, and North America (BASSETT et al. 1983; WILSON 1984), is probably a relatively recent introduction to Australia. Australian *Atriplex* species have been geographically isolated from those of Eurasia. The tropics of the East Indies are thought to have formed an effective barrier to gene flow (OSMOND et al. 1980). The only Australian species having possible affinity with European species is *A. australasica,* which is a C_3 plant (OSMOND 1974). However, there is some doubt as to its endemic status (WILSON 1984), and it is a tetraploid with 2n = 36 chromosomes (NOBS 1980): these two factors mitigated against its inclusion as male parent in this investigation.

Hybridization, both natural and artifical, has been well documented within the genus *Atriplex*. Evolutionary trends within the *A. prostrata* group in Scandinavia have been studied extensively (GUSTAFSSON 1973, 1976). In Britain, naturally occurring and artificial $C_3 \times C_3$ hybrids have been analyzed (TASCHEREAU 1986). The one intersectional $C_3 \times C_4$ cross attempted by TASCHEREAU (1986) was unsuccessful. These studies indicate that $C_3 \times C_3$ hybridization occurs readily in natural populations. Differing opinions have been expressed as regards $C_4 \times C_4$ hybridization: NOBS (1980) proposed that natural populations of diploid C_4 *Atri-*

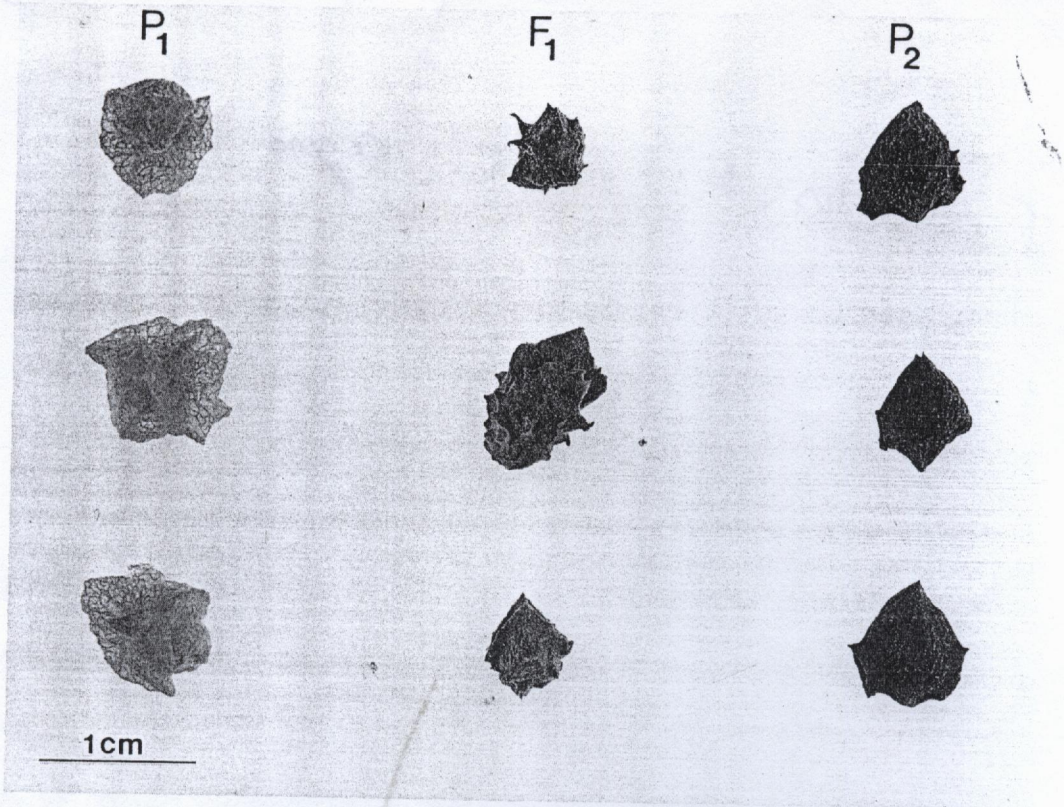

FIG. 3.—Fruit of *Atriplex lindleyi* subsp. *inflata* (P_1), *A. prostrata* (P_2), and their F_1 hybrid

plex species in Australia do not readily hybridize. However, WILSON (1984) suggested that crossing does occur, at least between subspecies of *A. lindleyi*. Spontaneous $C_4 \times C_3$ hybrids have not been observed, even between *A. rosea* and *A. triangularis*, whose distribution overlaps (OSMOND et al. 1980).

Ten percent of the *A. rosea* flowers pollinated by *A. prostrata* yielded F_1 seed (NOBS et al. 1970) while our crosses (table 1) gave much higher seed set. This suggests a closer affinity between the Australian C_4 species and *A. prostrata* than between *A. rosea* and *A. prostrata*. However, this may be a function of frequency and duration of artificial pollination. The fact that F_2 and subsequently F_3 and F_4 populations were obtained from the crosses to *A. rosea* (BJORKMAN et al. 1974), whereas we obtained very few F_2 seeds, indicates that *A. rosea* is more closely related genetically to *A. prostrata* than are any of the Australian C_4 species studied.

The two F_1 individuals which grew to maturity appeared to be hybrids, on the basis of their intermediate morphology, relative to the parents. The low viability of F_1 pollen and a seed set of only 12 full seeds in the 1,160 fruits analyzed from F_1 plants were also indicative of hybridity. It has been stated that low pollen fertility and low seed set may even occur in nonhybrid plants (BASSETT et al. 1983). We harvested many hundreds of fruit from parental plants which had been allowed to self. Those fruits rarely lacked seed, and pollen sampled from parental plants showed viability in excess of 95% (table 2).

Conventional hybridization has not resulted in the segregating diploid F_2 population which is required to analyze inheritance of C_4 photosynthesis in *Atriplex*. An alternative approach is now being undertaken: somatic hybridization of isolated protoplasts. Protoplasts have been isolated from C_4 and C_3 species of *Atriplex* (BIELIG and BROWNELL, unpublished). Somatic hybridization may overcome barriers to sexual incompatibility and result in larger F_1 and F_2 populations. Genomic incompatibility may, however, still be a barrier to interspecific hybridization.

Acknowledgments

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