

BIOLOGY OF OVER-WINTERING OF PERENNIAL ROOT
NODULES AND SEED DEVELOPMENT IN BEACH PEA
(*Lathyrus maritimus* L.)

CENTRE FOR NEWFOUNDLAND STUDIES

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**Biology of over-wintering of perennial root nodules and
seed development in beach pea (*Lathyrus maritimus* L.)**

By

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ABSTRACT

Beach pea (*Lathyrus maritimus* L.) is a potential cold-climate circumpolar legume crop that naturally grows along the shorelines of Newfoundland, Canada. The present study was undertaken to determine the over-wintering (winter survival) strategies of perennial root nodules of beach pea on the sandy beach of Salmon Cove, Newfoundland, with emphasis on oleosomes (lipid bodies). Seasonal changes in other biochemical constituents of nodules such as carbohydrates, proteins and elements were also studied. In addition, attempts were made to screen the suitable rhizobial inoculum for beach pea and also to trace the patterns of seed development, maturation and abortion in beach pea.

The native rhizobial strain (ACCCRC) isolated from beach pea nodules was found to be the most effective inoculum. It was superior in nitrogen-fixing traits compared to other bacterial strains. Studies on seasonal changes in nodule structure revealed that perennial nodules undergo winter dormancy when the aerial parts of the plant dry out. With the advent of spring, the nodule meristem is activated to regenerate effective nodule tissues that perform normal functions of the nodule in the following summer. A higher number of oleosomes was seen in histological preparations of winter nodules compared to summer nodules. As winter approached, nodules stored higher amounts of lipids, which decreased gradually throughout winter and spring. The degradation of oleosomes could be localized at the electron microscopic level.

Phospholipids and monoglycerides were identified as the major oleosomic lipids in perennial root nodules. C16:0, C18:0 and C18:1 were the dominant fatty acids. The fatty acid composition and double bond index differed among lipid classes depending

upon the season. Overall, the level of many unsaturated fatty acids increased and many saturated fatty acids decreased in oleosomes of winter nodules. Nodules selectively utilized fatty acids depending upon the season to overcome environmental influence.

Microscopical studies showed that nodule cells accumulated large amounts of amyloplasts with multiple starch grains in summer to be used in winter, and the degraded products could be visualized by electron microscopy. This was confirmed by quantitative data that starch content of nodules increased during the summer and decreased during the winter. Soluble sugars and non-reducing sugars increased in the winter, whereas reducing sugars increased in the fall. These results indicate that perennial nodules act as temporary storage organs. Nodules have large amounts of storage organelles such as oleosomes and amyloplasts during pre-winter months. These storage organelles may be catabolized to protect the nodule tissues from cold temperatures and also mobilized just before the growing season to support re-growth. Accumulation of more sugars in winter may help to maintain high osmolarity of cells that prevents freezing of dormant nodule tissues. Ultrastructural morphology of winter nodules confirms the lack of freezing damage.

Protein contents of nodules increased as the winter approached. A wide variation was observed in season-specific accumulation of amino acids and elements. Amino acids such as arginine, cystathionine, ethanolamine, histidine, hydroxyproline, ornithine and proline increased in winter nodules, whereas the level of γ -aminobutyric acid declined at the same time. Nodules collected in winter contained higher amounts of P, K, Ca, Mn, Cu and Zn, while summer nodules showed more Cl, Na, S, Mg, Al, Si, Mo and Fe. The possible involvement of some amino acids and elements in cold adaptation was

discussed. This study shows that perennial nodules of beach pea have complex winter survival strategies that involve modifications of anatomy, physiology and biochemistry of the nodules.

Generally, beach pea seeds showed almost a sigmoidal pattern of development. The stage 6 (S6) was identified as the physiological maturity stage in both beach pea and grass pea (*Lathyrus sativus* L.). This study suggests that harvesting of a seed crop when physiologically mature will ensure seeds of the best quality. Beach pea seeds exhibited precocious germination as well as hardseededness at S4. Hard seed coats completely prevented water absorption and germination at S5 and S6. In grass pea, precocious germination started at S3 and increased with seed maturity. There was no hardseededness in grass pea. Studies on seed abortion revealed that cryptic seed abortion was observed early in pod ontogeny in both beach pea and grass pea. Only 68% and 82% of ovules produced mature seeds in beach pea and grass pea, respectively. The patterns of seed abortion and development at different ovule positions within developing pods were random in beach pea and nonrandom in grass pea. Ovules in position 1 and 2 at the basal end of pods showed a greater probability of seed abortion in beach pea. Grass pea pods showed increased seed abortion towards the basal region of the pod.

Dedicated to my loving parents and sister

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TABLE OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGMENTS	vi
TABLE OF CONTENTS	viii
LIST OF TABLES	xiii
LIST OF FIGURES	xvi
LIST OF ABBREVIATIONS	xxi
CHAPTER I. GENERAL INTRODUCTION	1
1. 1. Beach pea	1
1. 1. 1. Taxonomy and biology of beach pea	1
1. 1. 2. Importance of beach pea	3
1. 1. 3. Nodules of beach pea	5
1. 1. 4. Seeds of beach pea	5
1. 2. Nitrogen fixation	6
1. 2. 1. Nitrogen cycle	6
1. 2. 2. Biological nitrogen fixation	8
1. 2. 2. 1. Asymbiotic nitrogen fixation	9
1. 2. 2. 2. Symbiotic nitrogen fixation	9
1. 2. 2. 3. <i>Rhizobium</i> -legume symbiosis	10
1. 2. 2. 3. 1. Nodule initiation	11
1. 2. 2. 3. 2. Nodule invasion	12
1. 2. 2. 3. 3. Nodule development and maturation	13
1. 2. 2. 3. 3. 1. Differentiation of uninfected cells	13
1. 2. 2. 3. 3. 2. Differentiation of infected cells	14
1. 2. 2. 3. 3. 3. Plant and rhizobial genes	15
1. 2. 2. 3. 4. Nodule anatomy	16
1. 2. 2. 3. 5. Nodule senescence	17
1. 2. 2. 3. 6. Nitrogen fixation	18
1. 2. 2. 3. 7. Nitrogen assimilation and transportation	19
1. 3. Oleosomes	20
1. 4. Cold adaptation	23
1. 5. Seed development and maturation	25

1. 6. Seed abortion	27
1. 7. Objectives of the present study	28

CHAPTER 2. NODULATION OF BEACH PEA INDUCED BY DIFFERENT STRAINS OF RHIZOBIA 31

2. 1. Abstract	31
2. 2. Introduction	31
2. 3. Materials and Methods	32
2. 3. 1. Plant materials	32
2. 3. 2. <i>Rhizobium</i> cultures	33
2. 3. 3. Nitrogen-fixing parameters	33
2. 3. 4. Microscopy and estimation of oleosomes	34
2. 3. 5. Statistical analysis	35
2. 4. Results and Discussion	35

CHAPTER 3. MICROSCOPICAL EVALUATION OF SEASONAL CHANGES IN PERENNIAL NODULES OF BEACH PEA: NODULE ANATOMY AND OLEOSOME (LIPID BODY) DISTRIBUTION 41

3. 1. Abstract	41
3. 2. Introduction	42
3. 3. Materials and Methods	42
3. 3. 1. Plant materials and sampling	42
3. 3. 2. Light and transmission electron microscopy	43
3. 3. 3. Micromorphometric assessment of oleosomes	43
3. 3. 4. Nodule histochemistry	44
3. 3. 5. Total lipid extraction and estimation	44
3. 3. 6. Statistical analysis	45
3. 4. Results	45
3. 5. Discussion	46

**CHAPTER 4. EFFECT OF SEASONAL CHANGES ON THE FATTY ACID
PROFILE OF DIFFERENT LIPIDS OF OLEOSOMES ISOLATED FROM
PERENNIAL NODULES OF BEACH PEA**

4. 1. Abstract	57
4. 2. Introduction	58
4. 3. Materials and Methods	58
4. 3. 1. Plant materials and sampling	58
4. 3. 2. Isolation of oleosomes	58
4. 3. 3. Separation of oleosomic lipids by thin layer chromatography	59
4. 3. 4. Analysis of fatty acid composition	60
4. 3. 5. Double bond index	61
4. 3. 6. Statistical analysis	61
4. 4. Results	61
4. 4. 1. Oleosomic lipids	61
4. 4. 2. Seasonal changes in fatty acid composition of PL	62
4. 4. 3. Seasonal changes in fatty acid composition of MG	62
4. 4. 4. Seasonal changes in fatty acid composition of DG	63
4. 4. 5. Seasonal changes in fatty acid composition of FFA	63
4. 4. 6. Seasonal changes in fatty acid composition of TG	63
4. 4. 7. Seasonal changes in DBI of oleosomic lipids	64
4. 5. Discussion	64

**CHAPTER 5. SEASONAL CHANGES IN CARBOHYDRATES OF PERENNIAL
NODULES OF BEACH PEA**

5. 1. Abstract	75
5. 2. Introduction	75
5. 3. Materials and Methods	76
5. 3. 1. Plant materials and sampling	76
5. 3. 2. Transmission electron microscopy	76
5. 3. 3. Extraction of carbohydrates	77
5. 3. 3. 1. Sugars	77
5. 3. 3. 2. Starch	77
5. 3. 4. Determination of carbohydrates	78
5. 3. 4. 1. Sugars	78
5. 3. 4. 1. 1. Reducing sugars	78
5. 3. 4. 1. 2. Total soluble sugars	79

5. 3. 4. 1. 3. Non-reducing sugars	79
5. 3. 4. 2. Starch	80
5. 3. 5. Statistical analysis	80
5. 4. Results	80
5. 5. Discussion	81

CHAPTER 6. SEASONAL CHANGES IN PROTEIN, AMINO ACID AND ELEMENTAL COMPOSITION OF PERENNIAL NODULES OF BEACH

PEA	90
6. 1. Abstract	90
6. 2. Introduction	91
6. 3. Materials and Methods	91
6. 3. 1. Plant materials and sampling	91
6. 3. 2. Extraction and estimation of total proteins	91
6. 3. 3. Analysis of total amino acid composition	92
6. 3. 4. Elemental analysis	93
6. 3. 5. Statistical analysis	94
6. 4. Results and Discussion	94
6. 4. 1. Total protein content	94
6. 4. 2. Total amino acid composition	95
6. 4. 3. Elemental composition	97

CHAPTER 7. CHANGES IN DEVELOPING SEEDS OF BEACH PEA AT SIX REPRODUCTIVE GROWTH STAGES

7. 1. Abstract	111
7. 2. Introduction	112
7. 3. Materials and Methods	112
7. 3. 1. Plant materials and sampling	112
7. 3. 1. 1. Beach pea	112
7. 3. 1. 2. Grass pea	112
7. 3. 2. Estimation of physical changes	113
7. 3. 2. 1. Fresh and dry weights of pod shells and seeds	113
7. 3. 2. 2. Moisture content of pod shells and seeds	113
7. 3. 3. Estimation of physiological changes	114
7. 3. 3. 1. Speed of germination	114

7. 3. 3. 2. Germination percentage	114
7. 3. 3. 3. Seedling length and dry weight	115
7. 3. 4. Estimation of biochemical changes	115
7. 3. 5. Microscopical observations of seed coat development in beach pea	115
7. 3. 5. 1. Light microscopy	115
7. 3. 5. 2. Scanning electron microscopy	116
7. 3. 6. Determination of hard seed coat development in beach pea..	116
7. 3. 6. 1. Water imbibition test	116
7. 3. 6. 2. Tetrazolium chloride test	117
7. 3. 7. Statistical analysis	117
7. 4. Results	118
7. 4. 1. Physical and physiological changes	118
7. 4. 2. Biochemical changes	119
7. 4. 3. Microscopical observations of beach pea seed coat development	119
7. 4. 4. Hard seed coat development in beach pea	120
7. 5. Discussion	121

**CHAPTER 8. EFFECT OF SEED ABORTION ON THE PATTERN OF SEED
FORMATION IN BEACH PEA**

8. 1. Abstract	140
8. 2. Introduction	140
8. 3. Materials and Methods	141
8. 3. 1. Plant materials and sampling	141
8. 3. 2. Measurements of pod and seed development	142
8. 3. 3. Frequency distribution of ovules and seeds per pod	142
8. 3. 4. Estimation of probability of seed abortion and development at different ovule positions	142
8. 3. 5. Statistical analysis	143
8. 4. Results	143
8. 5. Discussion	145

CHAPTER 9. GENERAL CONCLUSIONS 159

10. REFERENCES 163

LIST OF TABLES

<u>Table Number</u>		<u>Page</u>
CHAPTER 2		
Table 2. 1.	The list of rhizobial strains used	37
Table 2. 2.	Means (\pm SE) for various nitrogen-fixing parameters of beach pea and grass pea inoculated with different rhizobial strains	38
Table 2. 3.	The effect of rhizobial strains on oleosome content of beach pea and grass pea nodules at 9 weeks after inoculation	39
CHAPTER 3		
Table 3. 1.	Distribution of oleosomes in different nodule tissues of beach pea in summer and winter	50
CHAPTER 6		
Table 6. 1.	Seasonal changes in beach pea root nodules: significantly changing total amino acid ($\mu\text{g}/\text{mg}$ fresh mass)	101
Table 6. 2.	Seasonal changes in beach pea root nodules: non-significantly changing total amino acid and ammonia ($\mu\text{g}/\text{mg}$ fresh mass)	102
Table 6. 3.	Elemental composition (relative weight percentage) of different nodule tissues of beach pea: significantly distributed elements in winter and summer (P, Al, Si and Cu)	103

Table 6. 4.	Elemental composition (relative weight percentage) of different nodule tissues of beach pea: significantly distributed elements either in winter (Na, K and Mg) or summer (S)	104
Table 6. 5.	Elemental composition (relative weight percentage) of different nodule tissues of beach pea: non-significantly distributed elements in winter and summer (Cl, Mo and Ca)	105
Table 6. 6.	Elemental composition (relative weight percentage) of different nodule tissues of beach pea: non-significantly distributed elements in winter and summer (Mn, Fe and Zn)	106

CHAPTER 7

Table 7. 1.	Stages of seed development in beach pea based on the morphological characteristics of pods and seeds	126
Table 7. 2.	Stages of seed development in grass pea based on the morphological characteristics of pods and seeds	127
Table 7. 3.	Changes in the pod shell weight and moisture content of beach pea and grass pea at six reproductive growth stages	128
Table 7. 4.	Changes in the seed weight and moisture content of beach pea and grass pea at six reproductive growth stages	129
Table 7. 5.	Changes in the physiological vigour parameters of beach pea and grass pea seeds at six reproductive growth stages	130

Table 7. 6. Changes in total lipids and proteins of beach pea and grass pea seeds at six reproductive growth stages	131
---	-----

CHAPTER 8

Table 8. 1. Means (\pm SE) for pod growth and seed development in beach pea at six reproductive growth stages	148
Table 8. 2. Means (\pm SE) for pod growth and seed development in grass pea at six reproductive growth stages	149

LIST OF FIGURES

<u>Figure Number</u>		<u>Page</u>
CHAPTER 1		
Fig. 1. 1.	Beach pea plants during different seasons on the sandy beach of Salmon Cove, Newfoundland	29
Fig. 1. 2.	Photographs of beach pea plants in the field and laboratory	30
CHAPTER 2		
Fig. 2. 1.	Photomicrograph showing a part of a semi-thin section of nodule stained using the p-phenylenediamine technique	40
CHAPTER 3		
Fig. 3. 1.	Photomicrographs showing the distribution of oleosomes in different nodule tissues of beach pea	51
Fig. 3. 2.	Montage of photomicrographs showing meristem, invasion zone, symbiotic zone and senescent zone in indeterminate nodules of beach pea	52
Fig. 3. 3.	Photomicrographs showing changes in winter nodules	53
Fig. 3. 4.	Photomicrograph showing junction between the invasion zone and the early symbiotic zone in a winter nodule	54
Fig. 3. 5.	Photomicrographs of beach pea nodules showing oleosomes and the deposition of suberin	55

Fig. 3. 6.	Seasonal changes in total lipid content of root nodules of beach pea	56
------------	--	----

CHAPTER 4

Fig. 4. 1.	Thin layer chromatography of oleosomes isolated from perennial root nodules of beach pea	68
Fig. 4. 2.	Seasonal changes in the major oleosomic fatty acid composition of phospholipids (PL) isolated from perennial root nodules of beach pea	69
Fig. 4. 3.	Seasonal changes in the major oleosomic fatty acid composition of monoglycerides (MG) isolated from perennial root nodules of beach pea	70
Fig. 4. 4.	Seasonal changes in the major oleosomic fatty acid composition of diglycerides (DG) isolated from perennial root nodules of beach pea	71
Fig. 4. 5.	Seasonal changes in the major oleosomic fatty acid composition of free fatty acids (FFA) isolated from perennial root nodules of beach pea	72
Fig. 4. 6.	Seasonal changes in the major oleosomic fatty acid composition of triglycerides (TG) isolated from perennial root nodules of beach pea	73

Fig. 4. 7.	Seasonal changes in the double bond index of different oleosomic lipids isolated from perennial root nodules of beach pea	74
------------	---	----

CHAPTER 5

Fig. 5. 1.	Electronmicrograph of a beach pea nodule collected during August 1997 showing amyloplast with starch grains	84
Fig. 5. 2.	Electronmicrographs showing the changing patterns in the ultrastructure of amyoplasts of perennial root nodules in beach pea	85
Fig. 5. 3.	Seasonal changes in starch content of root nodules of beach pea .	86
Fig. 5. 4.	Seasonal changes in total soluble sugars of root nodules of beach pea	87
Fig. 5. 5.	Seasonal changes in non-reducing sugars of root nodules of beach pea	88
Fig. 5. 6.	Seasonal changes in reducing sugars of root nodules of beach pea	89

CHAPTER 6

Fig. 6. 1.	Photograph of a longitudinal section through a beach pea nodule showing different tissues	107
Fig. 6. 2.	Seasonal changes in total protein content of beach pea root	

	nodules	108
Fig. 6. 3.	Seasonal changes in elemental composition of beach pea nodules: elements which increase in winter	109
Fig. 6. 4.	Seasonal changes in elemental composition of beach pea nodules: elements which increase in summer	110

CHAPTER 7

Fig. 7. 1.	Six reproductive growth stages of pods and seeds in beach pea ...	132
Fig. 7. 2.	Six reproductive growth stages of pods and seeds in grass pea ...	133
Fig. 7. 3.	Cross section of beach pea seed at stage 2	134
Fig. 7. 4.	Beach pea seed at stage 3	135
Fig. 7. 5.	Photomicrograph showing cross section of beach pea seed at stage 5	136
Fig. 7. 6.	Structural features of mature beach pea seed at stage 6	137
Fig. 7. 7.	Pattern of water imbibition by beach pea seeds at different stages of development	138
Fig. 7. 8.	Photograph showing viability of ungerminated beach pea seeds of S4, S5 and S6 at 24 days after germination experiment	139

CHAPTER 8

Fig. 8. 1.	Beach pea pod collected at S4 showing aborted seeds, developing seeds, stylar end and basal end	150
------------	---	-----

Fig. 8. 2.	Frequency distribution of ovules per ovary in beach pea at six reproductive growth stages	151
Fig. 8. 3.	Shift in the frequency distribution of seeds per pod in beach pea at six reproductive growth stages	152
Fig. 8. 4.	Frequency distribution of ovules per ovary in grass pea at six reproductive growth stages	153
Fig. 8. 5.	Shift in the frequency distribution of seeds per pod in grass pea at six reproductive growth stages	154
Fig. 8. 6.	Probability of seed abortion at different ovule positions within a pod of beach pea at different growth stages	155
Fig. 8. 7.	Probability of seed abortion at different ovule positions within a pod of grass pea at different growth stages	156
Fig. 8. 8.	Probability of seed development at different ovule positions within a pod of beach pea at different growth stages	157
Fig. 8. 9.	Probability of seed development at different ovule positions within a pod of grass pea at different growth stages	158

LIST OF ABBREVIATIONS

ACCCRC	= Atlantic Cool Climate Crop Research Centre
Al	= Aluminium
ANOVA	= Analysis of Variance
ATP	= Adenosine Triphosphate
C:N	= Carbon:Nitrogen
Ca	= Calcium
Cl	= Chloride
Cu	= Copper
DBI	= Double Bond Index
DG	= Diglyceride
ER	= Endoplasmic Reticulum
Fe	= Iron
FFA	= Free Fatty Acid
ISTA	= International Seed Testing Association
K	= Potassium
kd	= Kilo Dalton
kV	= Kilo Volt
M	= Molarity
Mg	= Magnesium
MG	= Monoglyceride

Mn	= Manganese
Mo	= Molybdenum
Na	= Sodium
PBM	= Peribacteroid Membrane
PL	= Phospholipid
SE	= Standard Error
Si	= Silicon
SPSS	= Statistical Package for the Social Sciences
TG	= Triglyceride
TLC	= Thin Layer Chromatography
USDA	= United States Department of Agriculture
v/v	= Volume/Volume
w/v	= Weight/Volume
Zn	= Zinc

CHAPTER I

GENERAL INTRODUCTION

I. 1. Beach pea

I. 1. 1. Taxonomy and biology of beach pea

Beach pea (*Lathyrus maritimus* L.) is the main focus of the present study and grass pea (*Lathyrus sativus* L.) is used for comparison in some experiments. Both beach pea and grass pea are legume crops and belong to the family Leguminosae (Fabaceae), the subfamily Papilionoideae and the genus *Lathyrus*. The word “legume” is derived from the Latin term “legumen”, which means seeds harvested in pods. The enormous plant family, Leguminosae, with a worldwide distribution, has an estimated 16,000 to 19,000 species in about 750 genera. In economic importance, it is second only to the Gramineae (Poaceae); in size it is third only to the Orchidaceae and the Compositae (Allen and Allen, 1981). Only a few legume species are used by humans as food; common bean, field pea, chick pea, cow pea, green gram, black gram, lentil, pigeon pea, soybean and ground nut (Deshpande and Damodaran, 1990).

The word “*Lathyrus*” is derived from the Greek term “lathyros”, which means pea or pulse. There are about 130 species in the genus *Lathyrus* consisting of annuals and many climbing and herbaceous perennials. This genus is widespread in the temperate regions of both hemispheres where it occurs naturally (e.g. beach pea) and is cultivated (e.g. grass pea). *Lathyrus* species are well adapted to rather dry areas, yet tolerate water logging, grow well on poor land, and are resistant to cool weather. They occur in

meadows, along seashores, lakes and stream banks, roadsides and in thickets, fields and waste areas. Some *Lathyrus* species are used for soil cover, green manure, erosion control and rehabilitation of cut-over or burned-over land (Allen and Allen, 1981).

Beach pea, a nitrogen-fixing circumpolar legume, grows along the shorelines of arctic and subarctic regions of the world from Greenland to Siberia and Japan including Canada, USA and UK (Cooper, 1936; Fernald, 1950; Brightmore and White, 1963; Haskin, 1977; Randall, 1977; Donaldson and McMillan, 1981; Simon, 1988). In Canada, it is found in Newfoundland, Nova Scotia, Quebec, Ontario, British Columbia and the North West Territories (Hulten, 1968). This plant primarily colonizes maritime dunes but also occurs along the sandy shores of large fresh water lakes (Scoggan, 1978; Hitchcock, 1952; Lamoureux and Grandtner, 1977). On the Atlantic coast, it grows as far south as New Jersey, USA (Fernald, 1950; Scoggan, 1978; Erichson-Brown, 1979).

Beach pea is a glabrous perennial with a dark brownish-black taproot up to 2 m in length and erect semi-woody stock. Stems several, procumbent, striate, 2.5-3.5 mm in diameter; from 0.2 to 1.0 m or more in length. Leaves are slightly fleshy, pinnate, ending in a simple or branched tendril; leaflets are usually alternate but sometimes in 3 to 6 opposite pairs (Brightmore and White, 1963). Flowers are papilionate, 2.0-2.5 cm long and purple but change to violet before wilting (Asmussen, 1993). Pods are laterally compressed, pale brown, oblong, two-valved, 4-6 cm long, and dehiscent. Seeds are 3-10 in number per pod, more or less spherical, 4.0-4.5 mm in diameter with long hilum and greenish to chestnut brown (Brightmore and White, 1963).

Grass pea is a well-established, commercially-available, tropical semi-arid crop. The flowers of grass pea are papilionate and dull white, blue, pink or purple in colour. Pods are flat, dorsally broad with two ridges, short and 3-5 cm in length. Each pod contains 2-7 seeds. Mature seeds are rhomboid or triangular in shape, dull whitish gray brown and variously mottled (Chavan, 1998). Grass pea is a relatively productive crop compared to other pulses in regions characterized by poor soil (Duke et al., 1981). It is very well adapted to adverse climatic conditions and requires very little management for crop production. The seeds of this crop are a major source of protein for large sections of the population in Bangladesh, China, Ethiopia and India (Spencer et al., 1986; Kuo et al., 1994). In India and Java, grass pea is considered one of the most economical pulses for fodder and green manure in rice fields during the cool winter period (Allen and Allen, 1981). Moreover, its deep taproot and nitrogen-fixing ability make this crop an ideal choice for sustainable agriculture.

1. 1. 2. Importance of beach pea

The economy of Newfoundland depended on the fishery until recently when a decrease in fish populations in coastal regions caused the government to impose restrictions on catches. People are now looking for alternative sources of income. The low agricultural activity and high demand for forage and food grains has led to a search for a suitable crop for Newfoundland, where winters are long. Beach pea grows very well naturally on shorelines of Newfoundland without any crop management (Figs. 1. 1 and 1. 2). It is perennial and persistent for many years and resistant to frost and drought. It forms

large continuous stands by rhizomes and has prolific seed production (Martin et al., 1998c). Sometimes, the vegetative parts of beach pea are used as fodder for cattle (Bal and Barimah-Asare, 1993).

Recent trials in both greenhouse and field have shown promise in terms of growth characteristics of the plant (McKenzie and Donnelly, 1996) and the added advantage of the nutritional value of the seeds and other plant parts (Chavan, 1998) suggests that beach pea may be a good candidate as a cold-climate crop for food, feed or forage (McKenzie et al., 1997; Martin et al., 1998b). Thus, it could be developed into a profitable crop for Newfoundland farmers and industrial processors. Beach pea is also important because it fixes nitrogen with the help of symbiotic soil bacteria which allows it to grow in nutrient-poor areas that can be inhospitable to other plants (Bublitz, 1982; Barimah-Asare and Bal, 1994). The rhizobia-induced nodules of beach pea have been shown to fix atmospheric nitrogen at relatively low temperatures (Bal and Barimah-Asare, 1993).

Nodules and seeds are two important structures of the beach pea. The nodule is the source of combined nitrogen, which is important as a nitrogenous fertilizer for growth and reproduction. Seeds characteristically store relatively large amounts of food reserves, which support growth and development of the seedling during germination. These reserves are for the most part, but not exclusively, laid down as discrete, intracellular organelles and include lipid, protein, carbohydrate, organic phosphate and various inorganic compounds, depending upon the plant species. It is these storage materials that render seeds a valuable part of animal and human diets (Bewley and Black, 1978). Seeds are also important for propagation.

1. 1. 3. Nodules of beach pea

The bacteria isolated from nodules of beach pea are reported as fast growing *Rhizobium leguminosarum* biovar *vicia*, which have a wide range of pH and salt tolerance, and can infect *Vicia cracca*, but not *Vicia faba* and *Pisum sativum* (Barimah-Asare, 1991). According to Saw (1995), the mode of nodule infection in beach pea is not clear as neither infection threads through root hairs nor infection through cracks at the base of the lateral roots were observed. The slough-off region behind the root cap has been identified as a possible entry point for *Rhizobium*.

Beach pea nodules are perennial and indeterminate with the apical meristem capable of continuous growth (Barimah-Asare and Bal, 1994). Beach pea root nodules undergo dormancy over the cold winter months when the aerial parts of the plant dry out. With the advent of spring, the nodule meristem is activated to regenerate effective nodule tissues for subsequent nitrogen fixation. In a preliminary study, Bal and Khetmalas (1996) observed that the pre-winter nodules were filled with large populations of storage organelles such as oleosomes and amyloplasts. At present, no information is available about how the perennial nodules of beach pea survive the cold winter months along the shorelines of Newfoundland. In order to understand the winter survival strategies, it is important to study the seasonal changes in structure and the biochemical composition of perennial nodules of beach pea.

1. 1. 4. Seeds of beach pea

Beach pea seeds have not been used for food or feed purposes except during the

scarcity of other foods by stranded sailors (Fernald and Kinsey, 1958; Erichson-Brown, 1979). Since they have impermeable hard seed coats, either chemical scarification with concentrated sulphuric acid or physical scarification in the form of impaction in order to crack the seed coat have been recommended to enhance seed germination (Saw, 1995). The presence of neurotoxins has been reported as being at a very low concentration in beach pea compared to other legumes (Chavan, 1998).

Seeds of beach pea have substantially higher amounts of crude proteins, crude fibres, reducing sugars, total phenolics, ash and total free amino acids than seeds of grass pea and green pea (Chavan, 1998). Besides other biochemical compounds, such as lipids, carbohydrates, starch, minerals, tannins and antinutritional factors, in beach pea seeds, Chavan and his co-workers have also studied various biochemical constituents of other parts of the beach pea plant (Chavan, 1998; Chavan et al., 1999a,b,c; Shahidi et al., 1999). However, there is no information available on the pattern of seed development, maturation and abortion. Studying the pattern of seed development and maturation may enable us to understand the critical period of development and gain precise information on optimum time of harvest. This may help to harvest the seed crop in time without significant loss in seed quality and yield.

1. 2. Nitrogen fixation

1. 2. 1. Nitrogen cycle

Nitrogen, in its elemental gaseous form, constitutes 78% of the earth's atmosphere but the demand for fixed nitrogen by the biosphere exceeds its availability.

This is due to the fact that there are no nitrogen-fixing higher plants and eukaryotic organisms; only some prokaryotic organisms. The element, nitrogen, is essential for all living organisms because it is a component of many biomolecules. These include nucleic acids, proteins and amino acids, which occur in large amounts in all living cells, as well as quantitatively less significant compounds, such as coenzymes, vitamins and pigments (Gallon and Chaplin, 1987).

Nitrogen exists in several forms in our environment. The continuous interconversion of these forms by physical and biological processes constitutes the nitrogen cycle. Basically there are three different ways by which atmospheric nitrogen is fixed into a combined form (Gallon and Chaplin, 1987): (1) atmospheric nitrogen fixation – nitrogen reacts with oxygen due to lightning or ultraviolet radiation to form oxides that are carried to the soil with rainfall, (2) industrial nitrogen fixation (Haber Bosch process) – nitrogen and hydrogen react at high temperature (800 °C) and pressure (200 atm) to yield ammonia (Glenn and Dilworth, 1991) and (3) biological nitrogen fixation – the process by which atmospheric nitrogen is fixed into ammonia catalyzed by the nitrogenase enzyme complex in prokaryotic organisms.

In the nitrogen cycle, nitrogen compounds are added to soil or water and these compounds are then assimilated by plants and microorganisms. The assimilated nitrogen is returned to the soil on the death and decay of these organisms. Ammonia and nitrate are reversibly interchangeable within the soil by the action of various microorganisms. Animals are able to utilize only nitrogen compounds that have been previously assimilated by microbes and plants. Overall in this cycle, combined nitrogen can be lost

to the atmosphere by denitrification and gained from it by physical and biological nitrogen fixation processes (Gallon and Chaplin, 1987).

1. 2. 2. Biological nitrogen fixation

Biological nitrogen fixation involves highly specialized and intricately evolved interactions between soil microorganisms and higher plants for harnessing atmospheric elemental nitrogen (Shantharam and Mattoo, 1997) by reducing it to ammonia by the unique enzyme complex, nitrogenase. The ammonia produced can then be incorporated by enzymatic means into the growth and maintenance of the cell. The energy required for nitrogen fixation can be obtained either directly or indirectly from the oxidation of carbohydrates, which are produced by photosynthesis. Diazotrophic organisms are able to couple the oxidation of carbohydrates with the reduction of nitrogen (Gallon and Chaplin, 1987).

The ability to fix nitrogen seems to be exclusively the property of a limited number of prokaryotic species (Gallon and Chaplin, 1987), which usually have a mutual association with plants that benefits both partners. The nature of the association may be symbiotic, asymbiotic (free living) or in the form of casual associations with other organisms; they may be phototrophic or chemotrophic, autotrophic or heterotrophic; and they may fix nitrogen only under aerobic, anaerobic or microaerobic conditions (Lodha and Nainawatee, 1993).

1. 2. 2. 1. Asymbiotic nitrogen fixation

Asymbiotic nitrogen-fixing organisms include diverse group of prokaryotes, such as strict anaerobes, facultative anaerobes, microaerobes, obligate aerobes and some of the chemoautotrophs, cyanobacteria and photosynthetic bacteria (Gallon and Chaplin, 1987). Most of the free-living diazotrophic bacteria are heterotrophs with the exception of photosynthetic bacteria and cyanobacteria. They require habitats capable of providing a source of utilizable carbon substrates necessary to meet the energy demands of nitrogen fixation. For this reason, the rate of nitrogen fixed by free-living organisms is less than that of symbiotic bacteria (Evans and Burris, 1992; Hill, 1992). These organisms are found in both soil and aquatic environments. Nitrogen fixed asymbiotically is not directly available to plants until the nitrogen-fixing organisms die and decompose in the soil.

1. 2. 2. 2. Symbiotic nitrogen fixation

Symbiotic nitrogen fixation is a type of biological nitrogen fixation, where the bacteria mainly inhabit in specialized structures called nodules. Nitrogen-fixing symbioses fall into three main groups: (1) those involving interactions between legumes and soil bacteria of four genera, *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium*, collectively termed rhizobia (Whitehead and Day, 1997). Only one nonlegume plant, *Parasponia*, a member of family Ulmaceae, has been found to form symbiotic root nodules with *Bradyrhizobium* (Trinick, 1973; Evans and Burris, 1992). (2) those involving associations between nonleguminous angiosperms and the actinomycete, *Frankia* (Baker and Mullin, 1992; Evans and Burris, 1992). (3) those involving

cyanobacterial associations with some angiosperms, gymnosperms, pteridophytes, bryophytes and lichens (Lodha and Nainawatee, 1993). Although the vast majority of nodules occur on the roots of the host plant, certain aquatic and water-tolerant legumes produce nodules on their stems (Alazard, 1985) or trunk (Prin et al., 1991) in association with rhizobia. These nodules have been reported to be capable of high rates of nitrogen fixation (Subba Rao and Yatazawa, 1984).

1. 2. 2. 3. *Rhizobium* – legume symbiosis

A very common symbiotic association occurs between leguminous plants and rhizobia. Most members in the plant family Leguminosae (Fabaceae) form a symbiotic association with rhizobia, which plays a crucial ecological role in maintaining adequate nitrogen resources. This is achieved by the ability of rhizobia, which penetrate legume roots and elicit hypersensitive responses that lead to the appearance of nodules. Bacteria within these symbiotic structures fix atmospheric nitrogen and thus are of immense ecological and agricultural significance (Pueppke, 1996; Shantharam and Mattoo, 1997). In this symbiotic association, the rhizobia reduce atmospheric nitrogen into ammonia, which they export to the plant for assimilation; the plant reduces carbon dioxide into sugars during photosynthesis and translocates these to the root where bacteria use them as fuel for the high energy-demanding process of nitrogen fixation (Long, 1989).

1. 2. 2. 3. 1. Nodule initiation

Nodulation commences when the symbiotic partners encounter one another in the rhizosphere. Communication is established at this stage and is mediated by the exchange of diffusible chemical signals. Rhizobia are attracted to legume roots by phenolic compounds (flavonoids, flavanones, flavones and isoflavones) and betains in the root exudate (Maxwell and Phillips, 1990; Brewin, 1991; Kape et al., 1991; Kijne, 1992; Phillips et al., 1994), which stimulate the expression of nodulation (*nod*) genes of *Rhizobium* (Peters et al., 1986) and *Bradyrhizobium* (Kosslak et al., 1987). Some of these genes (Pueppke, 1996) code for bacterial signal molecules (lipochito-oligosaccharides) called *Nod* factors, which stimulate the expression of corresponding nodulation genes in the plant.

Legumes exude a characteristic spectrum of flavonoid compounds and *Nod* proteins from different species of rhizobia recognize particular flavonoids preferentially. This molecular recognition is an important determinant of host-*Rhizobium* specificity (Horvath et al., 1987; Spaink et al., 1989). After chemotaxis, infection is initiated when the bacteria come into contact with root hairs (Bhuvaneswari et al., 1980; Whitehead and Day, 1997).

A sulphated and acylated tetra-glucosamine glycolipid called *NodRm-I* has been identified as the secreted product of *nod* gene activity in *R. meliloti* (Lerouge et al., 1990). This signal molecule is responsible for several apparently unrelated plant responses, namely root hair curling, infection thread initiation and the induction of cell divisions in the cortical regions of alfalfa seedling roots (Brewin, 1991). Plant lectins and

rhizobial cell surface polysaccharides have also been reported to be involved in the specific attachment of rhizobia to the legume root hairs (Bohlool and Schmidt, 1976; Bal et al., 1978; Dazzo, 1978), however, lectins are likely to play a major role in nodule invasion rather than attachment of rhizobia (Roth and Stacey, 1991; Kijne, 1992).

As a result of *Rhizobium nod* gene activity (Dudley et al., 1987), there is an induction of mitotic activity in the cortex of root hairs (Calvert et al., 1984). Initially, the plane of cell division is oriented so that the axis of new wall deposition is perpendicular to the longitudinal axis of the root (anticlinal) resulting in the formation of a nodule primordium. Subsequently, this nodule primordium gives rise to an organized meristem consisting of a mass of small cells dividing in all planes. This mass of cells is in the outer cell layers in legumes with determinate nodules that lack meristems at maturity. They appear in deeper layers adjacent to the pericycle in species with indeterminate nodules that retain persistent meristems (Brewin, 1991).

1. 2. 2. 3. 2. Nodule invasion

Within a few hours of exposure to rhizobia, susceptible root hairs deform into a number of unusual shapes such as corkscrews, twins, twists, branches and spirals (Pueppke, 1996). The affected cells may even be enmeshed into a spaghetti-like mass (Haack, 1964). After physical attachment of rhizobia to the growing root hair surface, the deformed root hairs coil 360° and form the most severe degree of root hair curling known as “Shepherd’s crooks”. Root hair deformation is dependent on the presence of functional *Rhizobium nod* genes (Hirsch, 1992). After deformation, rhizobia enter the root via an

inward growth of the plant cell wall, known as the infection thread. The thread itself is roughly tubular in form and of plant origin (Pueppke, 1996). The infection thread grows through the root tissues until it reaches the cortex.

Infection threads are surrounded by a membrane known as the infection thread membrane, which is continuous with the plasma membrane of the infected cell (Newcomb, 1976; Verma et al., 1978). Bacteria are released from an unwalled area of the infection thread by endocytosis of the infection thread membrane into the cortical cells of the developing nodule. These cells are known as infected cells (Udvardi and Day, 1997; Whitehead and Day, 1997). The released bacteria are subsequently encapsulated by the host membrane (Patel and Yang, 1981; Roth and Stacey, 1989). The infected cells tend to stop dividing immediately, or soon after invasion by bacteria, and subsequently increase in size by expansion. As a consequence, infected cells are characteristically much larger than the uninfected nodule cells (Roth and Stacey, 1989).

1. 2. 2. 3. 3. Nodule development and maturation

1. 2. 2. 3. 3. 1. Differentiation of uninfected cells

Infection threads do not develop in the meristematic region. Perhaps the small and compact structure of these cells and the absence of a regular plane of cell division make the meristematic cells resistant to the development of infection threads. While some cells derived from the nodule meristem become infected by rhizobia, others remain uninfected and develop into a variety of specialized cell types. At a very early stage, nodule endodermis develops as a single layer of cells having suberized cell walls, dividing the

outer cortex from the central nodule parenchyma. Besides acting as a major barrier to the lateral diffusion of solutes, this impermeable sheath also influences the differentiation of neighbouring cells and tissues (Brewin, 1991). Below the nodule endodermis are several layers of uninfected cells, alternatively termed either the inner cortex or the uninfected nodule parenchyma (van de Wiel et al., 1990). Like the apical meristem, inner cortical cells are also tightly packed without intercellular air space that makes these cells the major barrier for oxygen diffusion (Parsons and Day, 1990).

A relatively small number of uninfected cells called interstitial cells are interspersed with the enlarged infected cells in the central region of the nodule. It seems these cells become metabolically specialized in ways that are very different from their infected counterparts (Scheres et al., 1990). These cells may represent a distribution network between the vascular tissue of the inner cortex and infected cells that conducts carbon substrates towards the nitrogen-fixing cells and organic nitrogen compounds away from this region (Brewin, 1991).

1. 2. 2. 3. 3. 2. Differentiation of infected cells

After entry of rhizobia into root cells, the formation of symbiosomes takes place, which become the fundamental nitrogen-fixing units of the nodule. The membrane and the bacteria within are together known as symbiosomes, a general term used to describe the symbiotic compartment of the endosymbiotic associates within the host cell (Roth et al., 1988). The membrane surrounding the bacterium in the legume nodule is called the peribacteroid membrane (PBM) and the space between the bacterium and PBM is called

the peribacteroid space (Udvardi and Day, 1997; Whitehead and Day, 1997). Only a few bacteria infect any one cortical cell and after endocytosis the bacteria proliferate, resulting in the cytosol of mature infected cells being packed with symbiosomes. In the nodules of temperate legumes, PBM division is coordinated with bacterial division, resulting in symbiosomes housing a single bacterium (Robertson and Lyttleton, 1984). In tropical legumes, the symbiosomes are multi-bacterial because PBM division is not so tightly coupled to that of the bacteria (Bergersen, 1982) and only after a certain point bacteria continue to divide (Goodchild and Bergersen, 1966).

Once the bacteria have stopped dividing, they undergo differentiation into their symbiotic form known as bacteroids (Bergersen, 1958). Differentiation involves the induction of genes necessary for the symbiotic state, including those encoding for nitrogenase. The surface of rhizobia possesses both outer and inner cell membranes as in other gram-negative bacteria (Vincent, 1977; Udvardi and Day, 1997). When inside the host cells, the rhizobia transform into nitrogen-fixing bacteroids with modifications in their outer membrane (Brussel et al., 1977).

1. 2. 2. 3. 3. Plant and rhizobial genes

The gene products of both the plant and the rhizobia play important roles in symbiosis. Plant genes are involved in almost all aspects of symbiosis, which include number and size of nodules, nodule morphogenesis and morphology and the rate of nitrogen fixation activity. The products of plant genes specifically expressed in the root nodules are known as 'nodulins' (Legocki and Verma, 1980). Nodulin genes are

differentially expressed during nodule development. Nodulin genes that are expressed at earlier stages of development are named 'early nodulins' and many of these early nodulins are expressed sequentially during nodule differentiation (Nap and Bisseling, 1990; Lodha and Nainawatee, 1993).

The nodulin genes expressed around the onset of nitrogen fixation are called 'late nodulins'. Leghemoglobin is the most abundant late nodulin, comprising almost 20% of the total soluble nodule proteins, and is an oxygen carrier in the infected cells of the nodule that facilitate the oxygen diffusion toward the bacteroids (Appleby, 1984; Lodha and Nainawatee, 1993). The bacterial genes responsible for symbiosis are collectively referred to as *sym* genes, which consist of *nod* (ability to nodulate), *nif* (nitrogen fixation) and *fix* (ability to fix nitrogen) genes (Lodha and Nainawatee, 1993).

1. 2. 2. 3. 4. Nodule anatomy

The morphology of legume root nodules shows considerable diversity (Corby, 1988) which reflects variation in the details of the symbiotic interaction for different species (de Faria et al., 1989). In general, root nodules of legumes are divided into two types; namely, indeterminate and determinate nodules (Sprent, 1979; Bergersen, 1982; Brewin, 1991). In indeterminate nodules, the meristem is apical and persistent, which gives an elongated or cylindrical and club-shaped appearance because new cells are constantly being added to the distal end of the nodule. This type of nodule may occasionally bifurcate to form a coralloid structure (Corby, 1988). Since an age gradient occurs from the distal meristem to the proximal point of attachment to the parent root, all

stages of nodule development can be seen in one nodule. For example, in *Medicago*, *Trifolium*, *Pisum* and *Vicia*, the indeterminate nodule is common (Brewin, 1991). In contrast, determinate nodules are spherical because meristematic activity is only a transient phase. Cell divisions cease early during nodule development and the final form of the nodule results from cell enlargement rather than cell division. Plant roots with determinate nodules include *Phaseolus*, *Glycine* and *Lotus* (Brewin, 1991; Hirsch, 1992).

1. 2. 2. 3. 5. Nodule senescence

Nodule tissues have a finite life span and their programmed senescence (apoptosis) is an integral part of the developmental sequence (Vasse et al., 1990). Indeterminate nodules break down in structure progressively from the base within a few weeks of initiation and, as nodules age, more of the nodule volume gradually becomes occupied with senesced or senescing tissue. In determinate nodules, senescence usually begins near the centre and spreads radially outwards with time. Senescence of nodules seems to begin with the infected cells losing ultrastructural features; changes in host nucleus and decay of bacteroids follow. Finally, bacteria enter the damaged cells from the infection thread remnants and there is usually a spread of bacteria between the cell walls, leading to intracellular masses of bacteria. Senesced tissue may serve as a reservoir of infection from which new nodule arises which over winter or are perennial (Bergersen, 1982).

The symbiosomes can be considered as a form of lysosomal compartment within the plant endomembrane system (Mellor, 1989). According to Brewin (1991), the

symbiosome has the apparent potential to develop into a lytic vesicle if the internal pH drops significantly and this would lead, directly or indirectly, to the degradation of bacteroids. The transport of dicarboxylic acid (Yang et al., 1990) and protons (Udvardi and Day, 1989) by the PBM allow for the maintenance of a proper pH in the peribacteroid space.

1. 2. 2. 3. 6. Nitrogen fixation

The basic requirements of biological fixation of dinitrogen include the nitrogenase enzyme complex, Mg^{2+} , ATP, a strong reducing agent and anaerobic conditions. Nitrogenase is expressed in rhizobia during symbiotic nitrogen fixation. It is composed of two oxygen-sensitive and easily-separable, non-haem iron proteins. The larger protein contains Mo, Fe and acid-labile sulphur and is called molybdenum-iron protein (MoFe protein) or molybdoferredoxin or azofermo. The smaller protein contains Fe and acid-labile sulphur and is called iron protein (Fe protein) or azoferredoxin or azofer. The Fe protein serves as one electron donor and a specific reductase for MoFe protein, and the latter binds and reduces dinitrogen. Therefore, Fe protein has been designated as 'dinitrogenase reductase' and MoFe protein as 'dinitrogen reductase' or 'true dinitrogenase' (Lodha and Nainawatee, 1993). The reduction of dinitrogen by nitrogenase can be represented as follows:



1. 2. 2. 3. 7. Nitrogen assimilation and transportation

The first stable product of biological nitrogen-fixation in legume nodules is ammonium (NH_4^+), which is excreted by bacteroids into the host cytoplasm where it is assimilated into organic molecules in the form of amino acids, amides or ureides. These products are transported via the xylem sap and are made available for plant growth. In general, tropical legumes are ureide exporters and temperate legumes are amide exporters based on the composition of the xylem fluid collected from excised nodules or nodulated root systems. The amide exporters transport asparagine (e.g. in *Trifolium repens* and *Glycine max*), glutamine (e.g. in *Lathyrus sativus* and *Pisum sativum*) or 4-methyleneglutamine (e.g. in *Arachis hypogaea*), while ureide exporters transport either allantoin and allantoic acid (e.g. in *Cajanus cajan* and *Cicer arietinum*) or citrulline (e.g. in *Alnus glutinosa* and *A. rubra*) (Lodha and Nainawatee, 1993).

The first organic compound, which is formed after NH_4^+ assimilation, is glutamine. The reaction is catalyzed by the combined activities of glutamine synthetase and glutamate synthase. The NH_4^+ incorporated into the amide position of glutamine is subsequently transferred to oxoglutarate in the reductive amination reaction (Meeks et al., 1978; Lodha and Nainawatee, 1993). Asparagine is probably superior to glutamine for nitrogen transport. It has a C:N ratio which favours a low energy cost in nitrogen transport. The synthesis of asparagine from aspartate takes place by glutamine-dependent asparagine synthetase. The 4-methyleneglutamine is synthesized from 4-methyleneglutamate by 4-methyleneglutamine synthetase (Lodha and Nainawatee, 1993).

Allantoin and allantoic acid are major ureides present in several tropical and subtropical legumes and they are energetically the most efficient nitrogen transport compounds. The C:N ratio of these compounds is 1:1 as compared with 1:0.50 of asparagine and citrulline, 1:0.40 of glutamine and 1:0.33 of 4-methyleneglutamine. Allantoin and allantoic acid are formed by the oxidative catabolism of purines. Citrulline is synthesized from ornithine and carbamoyl phosphate by ornithine carbamoyltransferase (Lodha and Nainawatee, 1993).

1. 3. Oleosomes

Most seeds contain storage lipids in the form of triglycerides (TG), which comprise 5-50% of the seed's total dry weight (Huang et al., 1993). Plant seeds store TG as energy sources for germination and postgerminative growth of seedlings. The storage TG is confined to discrete spherical organelles called oleosomes (Sorokin, 1967; Yatsu et al., 1971). They have been referred to variously as spherosomes (Frey-Wyssling et al., 1963; Sorokin, 1967), lipid-protein particles (Yatsu and Altschul, 1963), lipid vesicles (Mollenhauer and Totten, 1971), fat/lipid bodies (Lin and Huang, 1983) and oil bodies (Slack et al., 1980). The term, oleosome, has been used in the present text.

Most of the oleosomic studies in plants were carried out in seeds (Gurr et al., 1974; Huang, 1992; Huang et al., 1993; Murphy, 1993; Peng and Tzen, 1998). Oleosomes have also been reported in root nodules (Hameed and Bal, 1985; Newcomb and Wood, 1986; Bal et al., 1989; Jayaram and Bal, 1991; Barimah-Asare and Bal, 1994; Bal and Khetmalas, 1996), stem nodules (Bal and Denduluri, 1996; Denduluri and Bal,

1996) and pollen (Stanley and Linskens, 1974) of angiosperms as well as in tissues of some primitive plants, such as the megagametophytes of gymnosperms (Ching, 1970) and the spores of ferns (Gemrich, 1981). Like oleosomes, similar intracellular storage lipid organelles are also present in tissues of nonplant species, including brown adipose (Gurr, 1980) and other tissues of mammals (Fawcett, 1966; Murphy, 1990), eggs of some nematodes and other non-mammals (Rubin and Trelease, 1976) and other organisms such as yeast (Clausen et al., 1974; Murphy, 1990), fungi (Holdsworth et al., 1988), insects (McPherson and Kitchen, 1983) and algae (Roessler, 1988).

The isolated oleosomes from diverse plant species always assume a spherical shape and possess diameters of about 0.5-2.5 μm . The average size of oleosomes is species dependent and likely affected by nutritional and environmental factors. Within the same seed, oleosomes in different tissues may be of different sizes (Huang, 1992). The oleosome contains a TG matrix surrounded by a monolayer of phospholipids (PL) embedded with abundant proteins termed oleosins (Tzen et al., 1993; Khetmalas and Bal, 1997). Oleosins are alkaline proteins, unique to oleosomes, with low molecular masses of 15-26 kd depending on the species (Qu et al., 1986; Huang, 1992).

Oleosomes isolated from mature maize kernels contain 97% neutral lipids (mostly TG and a few percent of diglycerides), 1.4% oleosins, 0.9% PL and 0.09% free fatty acids (Huang et al., 1993). They are maintained as individual small organelles even after a long period of storage in plant seeds (Slack et al., 1980). This stability is a consequence of the steric hindrance and electronegative repulsion provided by oleosins on their surface (Tzen et al., 1992), therefore, these profound and compressed oleosomes in cells of a

mature seed never coalesce or aggregate. The physiological significance of maintaining a population of small discrete oleosomes is to provide ample surface area for the attachment of lipase to the organelles during postgerminative growth, so that the reserve TG can be mobilized rapidly.

The mechanism of oleosome formation in plants has long been and continues to be the subject of some controversy. Two models have been proposed to elucidate the ontogeny of oleosomes in developing seeds. One is the vesiculation or the budding oleosome model based on *in situ* electron microscopic observations (Schwarzenbach, 1971; Wanner et al., 1981). This model describes oleosome as being synthesized in the endoplasmic reticulum (ER) membrane. The TG synthesized in the ER is sequestered within the hydrophobic portion of the PL bilayer of the ER membrane, such that a nascent oleosome of a TG matrix surrounded by one PL layer is produced and it pinches off from the ER membrane. The other is the post-encasement model (Bergfeld et al., 1978; Huang, 1992; Murphy, 1993). According to this model, the oleosome would arise directly in the cytoplasm by condensation of TG molecules followed temporally by encasement with PL and oleosins. Nevertheless, the results of recent biochemical studies support the vesiculation model (Qu et al., 1986; Huang et al., 1993).

The presence of oleosomes in legume nodules indicates their possible involvement in nodule function and nitrogen fixation. In peanut root nodules, oleosomes are present in both infected and uninfected cells of nodules including senescent zone in all developmental stages. Morphometric analyses of oleosomes show high numbers in young differentiating nodules compared to mature nodules (Jayaram and Bal, 1991) when

high energy is needed for growth of host cells as well as proliferation of bacteroids (Bal and Denduluri, 1996). In mature nodules of peanut while nitrogen-fixation is active, oleosomes are still present but degradation of oleosomes occurs during photosynthate stress (Siddique and Bal, 1991, 1992).

It has been suggested that oleosomes in nodules of tropical legumes such as peanut serve as a supplementary source of energy apart from the direct supply of photosynthate to the bacteroids (Bal et al., 1989). In temperate legumes such as beach pea and *Oxytropis*, the oleosomes disappear in the symbiotic stage from infected cells, but remain in the nodule parenchyma. It has been suggested that the oleosomes in perennial temperate legumes serve as a cryoprotectant against low temperature stress (Newcomb and Wood, 1986; Bal and Khetmalas, 1996), a food reserve to be mobilized during the activation of the nodule meristem at the beginning of the growing season (Barimah-Asare and Bal, 1994) and as an additional source of energy for membrane proliferation and, growth and development of symbiosomes (Barimah-Asare and Bal, 1994; Prévost and Bal, 1994).

1. 4. Cold adaptation

Living organisms are in continuous contact with their environment and react to any fluctuations by changing their own physiological and morphological functions. The stress caused by a cold climate is one of the primary stresses limiting growth, productivity and the distribution of plants in the world. Prolonged exposure to cold causes various imbalances in plants, which include: decrease in the speed of chemical

reactions, reduction in the uptake of water and nutrients, loss of biosynthesis, reduction in assimilation and finally cessation of growth and cell death (Levitt, 1980; Larcher, 1995). In nature, plant cells tolerate cold stress by using various adaptation strategies. Cold adaptation at the cellular level is a complex phenomenon, which involves an amalgam of many events including alterations in the metabolism of proteins, carbohydrates, lipids, hormones and other compounds and also differential expression of various genes (Li and Christersson, 1993).

In response to cold, there is a general increase in the protein content of many plants (Graham and Patterson, 1982; Johnson-Flanagan and Singh, 1988) and also changes in many enzymes (Guy, 1990). The synthesis of specific proteins such as cold shock proteins (Guy et al., 1988), molecular chaperones (Guy et al., 1997) and antifreeze proteins or thermal hysteresis proteins (Urrutia et al., 1992; Griffith et al., 1997) has been reported in plants and they all seem to protect the cells against cold stress. Most plants adapted to survive cold stress have been found to increase the sugar content of tissues during cold acclimation (Levitt, 1980; Castonguay et al., 1997). Accumulation of sugars such as glucose, fructose, sucrose, trehalose, raffinose and stachyose has been reported to vary from species to species (Levitt, 1980). During cold adaptation, sugars act as a cryoprotectant by reducing freeze-induced dehydration of cells and stabilizing structural integrity of proteins, membranes and organelles (Levitt, 1980; Castonguay et al., 1997). The accumulation of sugars in cold-acclimated tissues is frequently accompanied by a concomitant decrease in starch reserves (Ristic and Ashworth, 1993), by hydrolysis, although they may also accumulate directly from photosynthesis (Levitt, 1980).

A very important key to survival in a cold climate involves the maintenance of adequate functioning of the cell membranes. Generally, membrane performance is, to a large extent, dependent on membrane fluidity and the phase behaviour of lipids, which are exquisitely sensitive to changes in ambient temperature (Hazel and Williams, 1990; Hazel, 1995). Plants can compensate for cold stress by changing the physical properties of membranes to ones appropriate to the circumstance.

1. 5. Seed development and maturation

The seed is normally the sexually-produced offspring in higher plants and the organ of dispersal. According to Abdul-Baki and Baker (1973), seed development is the period between fertilization and maximum fresh weight accumulation, and seed maturation begins at the end of seed development and continues up to harvest. During development and maturation, various morphological, physiological and biochemical changes occur in the seeds (Dure, 1975; Roti-Michelozzi et al., 1987; Bhattacharya and Saha, 1990; Sreeramulu et al., 1992; Bewley and Black, 1994; Gurusamy and Thiagarajan, 1998). The variations in pattern of seed development and maturation are numerous and depend upon the plant species and the environment through which the individual seeds develop.

In angiosperms, embryogenesis commences with double fertilization that gives rise to the zygote and the triploid cell that will give rise to endosperm tissue. These events take place within the embryo sac that is embedded in maternal tissue comprised of nucellus tissue and one or more integuments. This integumentary tissue is, in turn,

connected to the remainder of the ovary through the stalk-like funiculus (Dure, 1975). Seed development in angiosperms can be divided conveniently into three confluent stages. At first, during histodifferentiation, the zygote undergoes extensive mitotic division and the resultant cells differentiate to form the basic body plan of the embryo. At the same time, there is rapid development of the integument tissue, sometimes the nucellar tissue and the endosperm tissue surrounding the embryo (Dure, 1975; Kermode, 1990).

In the second stage, maturation occurs largely in the absence of further cell divisions and is characterized by cell expansion and deposition of reserves (normally proteins, along with lipids, carbohydrates, minerals, etc.) in the storage tissues (Bewley and Black, 1978, 1994). The endosperm continues to expand in size and cell number, which is followed later by embryo growth at the expense of the endosperm. The outer integument cells differentiate into the testa (seed coat) and in many cases the inner integument and nucellar cells are lost to the proliferating endosperm. Finally, desiccation sets in as the seed loses water to the surrounding environment and the seed coat tissue sclerifies and dies, encasing the endosperm and embryo well in a protective armour (Kermode, 1990). There is a gradation in the extent to which the developing embryo absorbs the nucellus/endosperm during embryogenesis throughout angiosperms, ranging from total absorption in legumes and cotton to almost no absorption in cereals (Dure, 1975).

Physiological maturity of seeds is normally understood to occur when the seed reaches its maximum dry weight (Harrington, 1972) beyond which nutrients are not

flowing from the mother plant into the seed. The vascular connection to the seed is broken by the formation of an abscission layer at physiological maturity (Eastin et al., 1973). Potential seed quality may be impaired when seeds are harvested while still developing on the mother plant (Pollock, 1972). Physiological deterioration might set in if the seeds are retained on the mother plant for a longer duration after physiological maturity (Gurusamy, 1999a). To reduce the risk of various biotic and abiotic hazards and the possible loss of yield and quality of seeds, harvesting of seeds at their physiological maturity is normally recommended for seed crops (Robertson et al., 1978; Gurusamy and Thiagarajan, 1998; Gurusamy, 1999a).

1. 6. Seed abortion

Pod and seed numbers are the most important components of yield variability in legume crops. Abortion of ovules and seeds within an ovary is a common occurrence in flowering plants. Many plant species regularly produce far more ovules than mature seeds (Wiens, 1984; O'Donnell and Bawa, 1993). The phenomenon of ovule/seed abortion is important because of its effects on plant fitness and its role in the evolution of other reproductive traits (Bawa et al., 1989).

Seed abortion is the mortality of immature seeds between fertilization and seed maturation (Bawa and Webb, 1984). Several multi-ovulated species are reported to show abortion of seeds before maturation (Nakamura and Stanton, 1987; Rocha and Stephenson, 1991; O'Donnell and Bawa, 1993; Mohan Raju et al., 1996; Gurusamy, 1999b). This has been explained by lack of pollination and/or fertilization (Zimmerman

and Pyke, 1988; Whelan and Goldingay, 1989), resource limitation (Lee and Bazzaz, 1986; Zimmerman and Pyke, 1988), predation (Mitchell, 1977; Herrera, 1984), genetic load (Wiens, 1984), sibling rivalry and parent-offspring conflict (Briggs et al., 1987; Ganeshiah and Uma Shaanker, 1988; Bawa et al., 1989) and neighbour effect (Joshi et al., 1993).

Seed abortion is also reported as a result of overproduction of ovules as a bet-hedging strategy to overcome the unfavourable conditions (Ehrlen, 1991) and an adaptive strategy to gain dispersal advantage (Augspurger and Hogan, 1983; Ganeshiah and Uma Shaanker, 1988; Mohan Raju et al., 1995). In many plants, the pattern of seed abortion within developing fruits is nonrandom (Bawa and Webb, 1984; O'Donnell and Bawa, 1993). Four patterns of seed abortion have been described. Abortion occurs either at (1) the base of the fruit (O'Donnell and Bawa, 1993), (2) the stylar end (Marshall and Ellstrand, 1988), (3) both ends of the fruit (Linck, 1961; Mazer et al., 1986; Hossaert and Valero, 1988), or at (4) other positions (Horovitz et al., 1976).

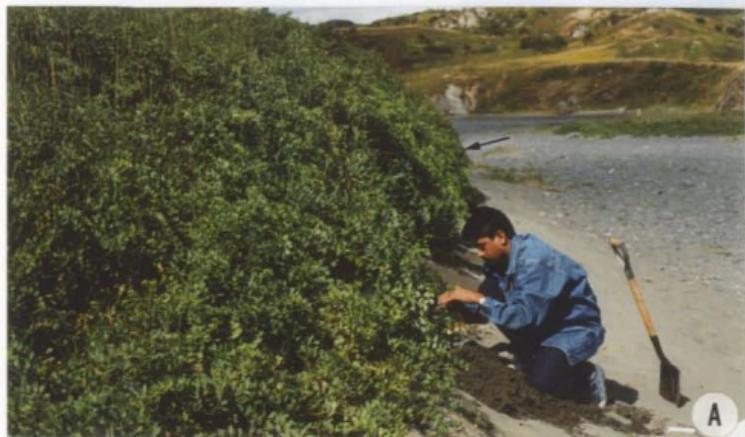
1. 7. Objectives of the present study

The objectives of present study are: (1) to select the most suitable rhizobial strain for symbiosis in beach pea, (2) to evaluate how the perennial root nodules survive during winter and (3) to determine the patterns of seed development, maturation and abortion.

Fig. 1. 1. Beach pea plants during different seasons on the sandy beach of Salmon Cove, Newfoundland. A. Winter view (January 1998). Note the ground is covered with snow (arrows) and there are no aerial parts of plant. B. Regenerating plants (arrows) from underground rhizomes during early spring (April 1998). C. Summer view (June 1998). Note the vigorous plants (arrows).



Fig. 1. 2. Photographs of beach pea plants in the field and laboratory. A. Vigorously growing beach pea plants (arrow) during summer season on Salmon Cove's sandy beach. B. Beach pea experimental plot during the fall on Salmon Cove's sandy beach. C. Beach pea plants in pots, grown from plants obtained from sandy beach of Salmon Cove and left out doors for winter sampling.



CHAPTER 2

NODULATION OF BEACH PEA INDUCED BY DIFFERENT STRAINS OF RHIZOBIA

2. 1. Abstract

In an attempt to screen the most effective rhizobial strain for beach pea, rhizobia from 8 different species of *Lathyrus* were tested along with the native strain for 9 weeks in pot culture. The native strain, ACCCRC, isolated from beach pea proved to be the most effective. The tropical legume, grass pea, tested with the above strains failed to nodulate with ACCCRC, USDA 2422 and USDA 2446. Oleosome content of nodules assessed from histological sections revealed higher numbers in beach pea than in grass pea. The number of oleosomes in nodules did not correlate with nitrogen-fixing parameters.

2. 2. Introduction

Effective symbiosis can only be achieved when the nodules are formed by efficient and effective rhizobia. After rhizobial strains have been isolated from nodules, they must be evaluated for their ability to form nodules and fix nitrogen with targeted legumes. The source of rhizobial strains for a strain selection programme can range from local isolates to cultures isolated from other species (Somasegaran and Hoben, 1994).

Strains of rhizobia have been isolated from the root nodules of beach pea populations (Martin et al., 1998c). In the present study, strains isolated from other species of *Lathyrus* have been evaluated along with the local strain for suitability as inocula for

beach pea by assessing nodulation and effectiveness in terms of plant colour, fresh weight and dry weight of plants. An attempt was also made to relate the results to morphometric estimation of oleosomes in histological sections of the nodules.

2. 3. Materials and Methods

2. 3. 1. Plant materials

Beach pea seeds were collected from naturally-growing stands on Bellevue Beach, Newfoundland. The seeds were mechanically scarified by rotating them between two rough surfaces of a petri dish coated with sand paper at the rate of 30 rotations per minute per 50 seeds for 2 minutes. For comparison, the well-established, commercially-available grass pea, a tropical semi-arid crop, was also used. The grass pea seeds germinated without any treatment. After surface sterilization, the seeds of beach pea and grass pea were placed in separate sterile petri dishes with pre-sterilized moistened filter paper. After 6 days in the dark, the germinated seeds were planted in sterilized pots containing sterilized vermiculite. The seedlings were separately inoculated with 9 different rhizobial strains containing 2950×10^5 cells/ml of yeast extract mannitol broth at the rate of 2 ml per seedling and allowed to grow in controlled environment chambers (Conviron S10H, Controlled Environments Ltd., Manitoba, Canada) with a day/night period of 16 hours/8 hours at 16 °C/8 °C and irrigated with a nitrogen-free nutrient solution (Eilffolk, 1960).

2. 3. 2. *Rhizobium* cultures

Rhizobium cultures, except ACCCRC, listed in Table 2. 1 were obtained through the courtesy of Dr. Peter Van Berkum, Soybean and Alfalfa Research Laboratory, USDA, Beltsville Agricultural Research Centre, Maryland, USA. The cultures were maintained in yeast extract mannitol agar and in a broth containing 10 g mannitol, 0.5 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.4 g yeast extract in 1 litre of distilled water at pH 6.8 - 7.0 with constant shaking in an Orbit Environs Shaker (Lab - Line Inc., Illinois, USA) at 28 °C (Vincent, 1970). The local strain (ACCCRC) was kindly provided by Dr. David B. McKenzie, Atlantic Cool Climate Crop Research Centre (ACCCRC), St. John's, Newfoundland, Canada. Martin et al. (1998c) have isolated this local strain from the root nodules of beach pea growing naturally in Bellevue Beach, Newfoundland.

2. 3. 3. Nitrogen-fixing parameters

Nine weeks after inoculation, the plants were harvested. Plant colour was rated on a scale of 1 to 3; 1 = green, 2 = green + yellow and 3 = yellow following Wynne et al. (1980). The total number of nodules per plant was counted. After measuring fresh weight with a Mettler PE 360 electronic balance (Mettler Instrumente AG, Zurich, Switzerland), the plants were dried in a Boekel 01785 oven (Boekel, Philadelphia, USA) at 110 °C for 48 hours. After cooling for 30 minutes at room temperature, the dry weight of plants was recorded.

2. 3. 4. Microscopy and estimation of oleosomes

Beach pea and grass pea nodules were sliced with a clean razor blade and fixed in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde (Karnovsky, 1965) in 0.1 M Sorensen's phosphate buffer (pH 7.2) for 60 minutes at 20 °C. After fixation the slices were washed with the same buffer at least three times, followed by treatment with 1% osmium tetroxide (OsO₄) in the same buffer for 60 minutes at 4 °C. The samples were washed and dehydrated in an ethanol series and embedded in Spurr's resin (Spurr, 1969). For *en bloc* staining of oleosomes, samples were treated with p-phenylenediamine in 70% ethanol for 60 minutes during dehydration (Bal, 1990). For precise localization of oleosomes, the control samples were treated with hexane for 45 minutes after fixation and dehydration without OsO₄ treatment as described in Bal (1990). Semi-thin, 1.5 µm, sections were cut with a Sorvall® "Porter-Blum" MT-1 ultramicrotome (Ivan Sorvall Inc., Connecticut, USA). Photomicrographs were taken with a Zeiss AXIOMAT photomicroscope (Carl Zeiss, West Germany) without further staining. From photomicrographs, in order to arrive at the number of oleosomes per unit area, random counts were made from oleosome containing areas. Figure 2. 1 is shown as an example from beach pea nodule induced by ACCRC strain. The tissue was stained specifically for oleosomes, which appear as dark brown particles in the cytoplasm.

2. 3. 5. Statistical analysis

For all sets of data, one-way analysis of variance (ANOVA) was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test at $P = 0.05$.

2. 4. Results and Discussion

Final evaluation of the symbiosis was based on several measurable parameters. The dry weight of plants is the best and generally accepted criterion for nitrogen-fixing effectiveness (Somasegaran and Hoben, 1994). Wynne et al. (1980) reported that plant colour and dry weight are excellent parameters to estimate strain efficiency and acetylene reduction measurements are not as useful as plant colour or dry weight. In the present study, the local strain, ACCCRC, outperformed all the USDA strains in beach pea (Table 2. 2). Plants of this strain were greener, weighed more in both fresh and dry conditions, and produced a higher number of nodules. This indicates the effectiveness of this rhizobial strain. Martin et al. (1998c) have also reported that the local strain (ACCCRC) produced higher seed yield compared to three Quebec strains in field trials. In grass pea, the most effective strains were USDA 2414 and 2411, which recorded more fresh and dry weights of plants and nodule numbers. All rhizobial strains used in this study induced nodulation in beach pea. ACCCRC, USDA 2422 and 2446 failed to produce nodules in grass pea.

Beach pea nodules contained more oleosomes than the grass pea nodules (Table 2. 3). The number of oleosomes in the nodules was highest in plants inoculated with USDA 2422 followed by USDA 2416 and 2446 in beach pea, whereas grass pea plants inoculated

with USDA 2416 showed the highest number of oleosomes. It has been shown that persistent oleosomes in the infected cells of the symbiotic nodules are utilized as a supplementary source of energy and carbon during nitrogen fixation in peanut (Bal and Siddique, 1991; Siddique and Bal, 1991, 1992). In beach pea, while oleosomes are present in the nodule parenchyma, vascular tissue and uninfected interstitial cells, they are absent in the mature infected symbiotic cells (Fig. 2. 1). As pointed out by Barimah-Asare and Bal (1994), oleosomes are not involved in nitrogen fixation *per se* in beach pea, but they may serve as an energy reserve for over-wintering (Bal and Khetmalas, 1996). In the present investigation also, nitrogen-fixing parameters do not correlate with the number of oleosomes in the nodule. They more likely participate in the protection of the nodule tissues during dormancy and winter stress in a cold-climate adapted plant like beach pea. Similar accumulation of oleosomes in 10 weeks old alfalfa nodules corroborates these data (Martin et al., 1998a).

In conclusion, the local strain (ACCCRC) is the best suitable rhizobial inoculum for beach pea and strains USDA 2414 and 2411 are effective inocula for grass pea.

Table 2. 1. The list of rhizobial strains used.

Rhizobial strains	Source
ACCCRC*	<i>Lathyrus maritimus</i>
USDA 2422	<i>Lathyrus cicera</i>
USDA 2411	<i>Lathyrus tuberosus</i>
USDA 2444	<i>Lathyrus japonicus</i>
USDA 2446	<i>Lathyrus latifolius</i>
USDA 2410	<i>Lathyrus sphaericus</i>
USDA 2416	<i>Lathyrus ochroleucus</i>
USDA 2417	<i>Lathyrus hirsutus</i>
USDA 2414	<i>Lathyrus polymorphus</i>

*Isolated from root nodules of beach pea growing in Bellevue Beach, Newfoundland, Canada.

Table 2. 2. Means (\pm SE) for various nitrogen-fixing parameters of beach pea and grass pea inoculated with different rhizobial strains.

Rhizobial Strains	Plant color ^a		Fresh weight (mg/plant)		Dry weight (mg/plant)		Number of nodules per plant	
	Beach pea	Grass pea	Beach pea	Grass pea	Beach pea	Grass pea	Beach pea	Grass pea
ACCCRC	1.00 \pm 0.00 ^a	3.00 \pm 0.00 ^b	1302.00 \pm 571.70 ^a	284.00 \pm 10.40 ^d	226.70 \pm 82.20 ^b	75.70 \pm 6.94 ^b	161.00 \pm 29.14 ^a	0.00 \pm 0.00 ^e
USDA 2422	1.33 \pm 0.33 ^{ab}	3.00 \pm 0.00 ^b	282.33 \pm 88.82 ^b	301.33 \pm 37.70 ^{cd}	70.70 \pm 21.65 ^b	115.33 \pm 11.72 ^{ab}	19.33 \pm 9.10 ^b	0.00 \pm 0.00 ^e
USDA 2411	1.33 \pm 0.33 ^{ab}	1.33 \pm 0.33 ^a	532.70 \pm 100.20 ^b	1007.33 \pm 145.70 ^{ab}	86.70 \pm 19.64 ^b	199.00 \pm 26.84 ^a	27.70 \pm 2.90 ^b	53.33 \pm 8.84 ^{ab}
USDA 2444	1.67 \pm 0.33 ^{ab}	1.33 \pm 0.33 ^a	485.70 \pm 36.10 ^b	416.33 \pm 44.40 ^{cd}	94.00 \pm 2.90 ^b	150.70 \pm 8.80 ^{ab}	31.00 \pm 1.20 ^b	11.70 \pm 1.80 ^{bc}
USDA 2446	1.00 \pm 0.00 ^a	2.33 \pm 0.33 ^{ab}	404.00 \pm 44.51 ^b	382.33 \pm 41.60 ^{cd}	84.00 \pm 8.20 ^b	116.33 \pm 9.40 ^{ab}	47.00 \pm 20.13 ^b	0.00 \pm 0.00 ^e
USDA 2410	1.67 \pm 0.33 ^{ab}	1.67 \pm 0.67 ^{ab}	275.70 \pm 6.10 ^b	641.70 \pm 46.74 ^{bcd}	53.00 \pm 1.20 ^b	168.70 \pm 21.73 ^a	28.70 \pm 1.50 ^b	47.33 \pm 18.02 ^{ab}
USDA 2416	3.00 \pm 0.00 ^c	2.33 \pm 0.67 ^{ab}	396.33 \pm 52.54 ^b	828.00 \pm 191.94 ^{abc}	58.70 \pm 11.30 ^b	159.70 \pm 22.80 ^{ab}	17.50 \pm 0.90 ^b	29.33 \pm 5.84 ^{abc}
USDA 2417	1.00 \pm 0.00 ^a	2.00 \pm 0.58 ^{ab}	457.00 \pm 19.10 ^b	974.00 \pm 389.50 ^{ab}	81.70 \pm 0.33 ^b	175.00 \pm 69.94 ^a	47.00 \pm 0.60 ^b	44.00 \pm 31.30 ^{abc}
USDA 2414	2.00 \pm 0.58 ^b	1.00 \pm 0.00 ^a	207.70 \pm 6.64 ^b	1270.00 \pm 125.71 ^a	48.70 \pm 2.60 ^b	170.33 \pm 4.33 ^a	21.00 \pm 1.20 ^b	72.70 \pm 17.84 ^a

^aRated with 1 = green, 2 = green + yellow and 3 = yellow.

Values are means (\pm SE) of three replications.

Means (\pm SE) followed by different letters in the same column are significantly different at $P = 0.05$ using Duncan's multiple comparison test.

Table 2. 3. The effect of rhizobial strains on oleosome content of beach pea and grass pea nodules at 9 weeks after inoculation.

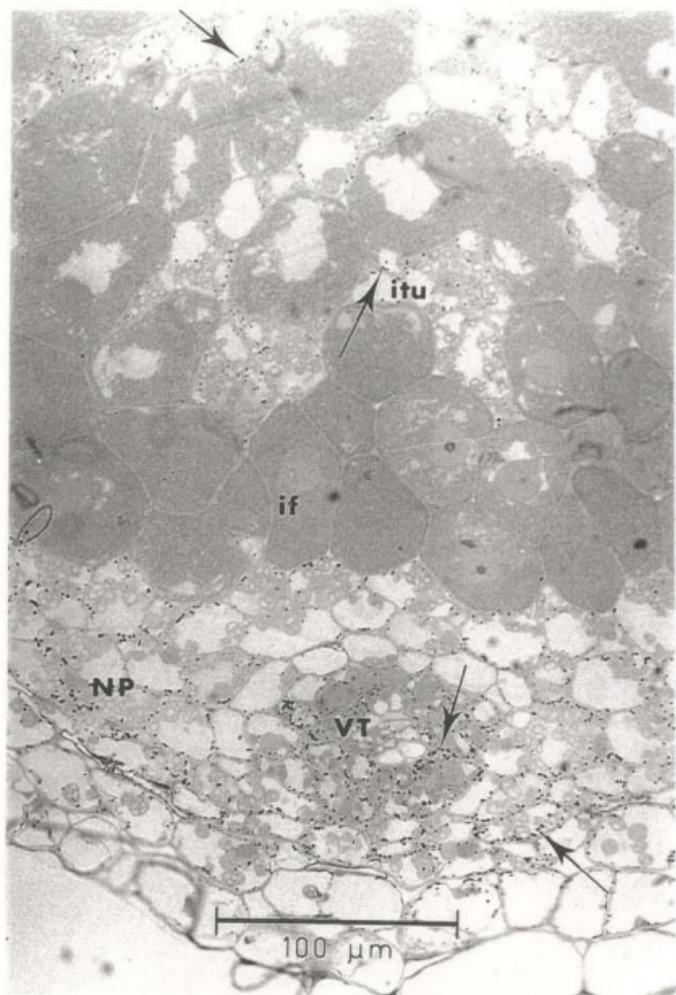
Rhizobial strains	Number of oleosomes ($\times 10^{-3} \mu\text{m}^{-2}$ of $1.5 \mu\text{m}$ semi-thin sections)	
	Beach pea	Grass pea
ACCCRC	6.94 ± 0.57^c	*
USDA 2422	12.10 ± 0.83^a	*
USDA 2411	6.24 ± 0.39^{cd}	1.74 ± 0.35^b
USDA 2444	2.92 ± 0.19^e	2.50 ± 0.12^b
USDA 2446	7.40 ± 0.71^{bc}	*
USDA 2410	6.54 ± 0.69^{cd}	1.50 ± 0.37^b
USDA 2416	9.10 ± 0.54^b	5.40 ± 1.50^a
USDA 2417	4.70 ± 0.79^d	2.10 ± 0.15^b
USDA 2414	5.84 ± 0.59^{cd}	1.90 ± 0.29^b

*No oleosome count was done due to the absence of nodulation.

Values are means (\pm SE) of five replications.

Means (\pm SE) followed by different letters in the same column are significantly different at $P = 0.05$ using Duncan's multiple comparison test.

Fig. 2. 1. Photomicrograph showing a part of a semi-thin section of nodule stained using the p-phenylenediamine technique. The arrows indicate oleosomes as dark-stained particles in the nodule parenchyma (NP), vascular tissue (VT) and uninfected interstitial cells (itu) in the symbiotic zone. Note the absence of oleosomes in infected symbiotic cells (if).



CHAPTER 3

**MICROSCOPICAL EVALUATION OF SEASONAL CHANGES IN
PERENNIAL NODULES OF BEACH PEA: NODULE ANATOMY
AND OLEOSOME (LIPID BODY) DISTRIBUTION**

3. 1. Abstract

Perennial nodules of beach pea undergo winter dormancy and become active in the spring. The nodules sampled in winter show dormant tissue and bacteria without any loss of structural integrity. In the senescent zone, bacteroids are found to be disintegrated. Oleosomes are clearly localized in the meristem, invasion zone, interstitial cells of the symbiotic and senescent zones, vascular tissue and cortical cells of both winter and summer nodules. In all the tissues, significantly larger numbers of oleosomes are seen in histological preparations of nodules sampled in winter compared with nodules sampled in summer. As winter approaches, the nodules show an increasing accumulation of lipids. The nodule tissues are protected from winter stress and pathogen attack by suberized peridermal cells. It may be concluded that oleosomes may be considered as a multipurpose organelle, which release energy for various metabolism in the cells, prevent freezing in the localized microenvironment of the cells, insulate the cells and supply fatty acids for membrane synthesis during cell growth.

3. 2. Introduction

Perennial legumes of arctic/subarctic regions have symbiotic nitrogen-fixing nodules, which allow them to grow in nutrient-poor soils. Earlier reports on perennial nodules have clearly recorded seasonal growth and activity by the presence of constrictions which apparently reflect periods of dormancy and growth (Pate, 1961; Bergersen et al., 1963).

In a preliminary study, Bal and Khetmalas (1996) have shown the histological status of the pre-winter nodules in beach pea, which indicated the formation of a 'sink' in the nodule tissue where storage organelles are deposited in preparation for the winter season. The present investigation has been extended to biochemical and ultra-structural studies of the perennial nodules throughout the year, including the winter months, with special reference to lipids and oleosomes, in particular.

3. 3. Material and Methods

3. 3. 1. Plant materials and sampling

Beach pea plants growing naturally on the sandy beach of Salmon Cove, Newfoundland, were sampled for nodules throughout the year [fall (September-December), winter (January-April), spring (April-May) and summer (June-August)] in the present study except in winter, when the ground was frozen. In winter months, plants grown in pots were kept outdoors and sampling was possible after thawing the pots for 18 hours at 5-10 °C in the greenhouse.

Nodules were collected by gently removing the soil and exposing the rhizomatous stem with adventitious roots and nitrogen-fixing nodules. Collection dates were August 28, 1997; September 27, 1997; October 25, 1997; November 16, 1997; December 18, 1997; January 21, 1998; February 20, 1998; March 18, 1998; April 27, 1998; May 9, 1998; June 15, 1998; July 11, 1998 and August 3, 1998. The nodules collected at various seasons were frozen at -20°C until further biochemical analysis could be done.

3. 3. 2. Light and transmission electron microscopy

The sample preparation and light microscopy were carried out as described in chapter 2 (see section 2. 3. 4 on page number 34). For transmission electron microscopy, ultra-thin sections were cut with a Sorvall MT-1 ultramicrotome. A Zeiss109 electron microscope (Carl Zeiss, West Germany) at 60 kV, was used for observation after staining with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

3. 3. 3. Micromorphometric assessment of oleosomes

From photomicrographs of different nodule tissues, in order to arrive at the number of oleosomes per unit area, random counts were made from oleosome-containing areas. The distribution of oleosomes in different nodule tissues of beach pea is shown in figure 3. 1.

3. 3. 4. Nodule histochemistry

Histochemical tests for suberin were performed on fresh hand-cut sections by treating with a saturated solution of sudan III in 70% ethanol for 3-5 hours. The sections were then quickly rinsed with 70% ethanol, mounted in a drop of melted glycerine jelly and observed with bright-field microscope (O'Brien and McCully, 1981). Fresh razor-cut sections were mounted in 0.02M phosphate buffer at pH 9.1 and observed for autofluorescence of suberin using a Nikon E400 fluorescence microscope (Nikon, Japan) at wavelength range of 330 - 380 nm with 420 nm barrier filter. Control sections were mounted in concentrated sulphuric acid to test the resistance to autofluorescence (Gahan, 1984).

3. 3. 5. Total lipid extraction and estimation

The total lipid was determined by the gravimetric method (Bligh and Dyer, 1959). One gram of nodules (fresh mass) from different seasons was homogenized separately in 10 ml of 50mM trisHCl buffer containing 0.5M NaCl at pH 7.2 on ice. The homogenate was combined with a mixture of chloroform and methanol in a ratio of 1.25:2.25 (v/v) to extract lipids. The chloroform layer was separated by centrifugation using Sorvall® RC 5C Plus centrifuge (Sorvall Instruments, Connecticut, USA) at 5,000 g for 20 minutes and allowed to stand over night after combining with a mixture of chloroform and distilled water in a ratio of 1:1 (v/v). The chloroform layer separated from water was collected in a pre-weighed vial for evaporation under nitrogen gas. The vial with the lipid residue was weighed again to estimate the amount of total lipid.

3. 3. 6. Statistical analysis

For all data sets, one-way analysis of variance was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test at $P = 0.05$.

3. 4. Results

Structural changes in summer samples of a mature nodule can be compared with samples from early spring that have undergone a full dormancy over winter (Fig. 3. 2). Nodule sections from both seasons clearly showed the different regions such as the meristem, the invasion zone, the symbiotic zone which is pink in colour from leghemoglobin, and the senescent zone. The senescent zone is very short in the mature nodule (Fig. 3. 2A). During early spring in the month of April, when the aerial parts of the plants were still not above ground, the nodules start to regenerate and they also show the past years long senescent zone and the newly developing pink coloured symbiotic zone (Fig. 3. 2B).

Samples taken in the winter months of January and February showed dormant nodule tissues without a symbiotic zone (Fig. 3. 3A). The nodule meristem and invasion zone contained oleosomes and amyloplasts (Figs. 3. 3B and 3. 3C). The persistent infection threads were observed in the invasion zone with bacteria surrounded by extracellular matrix, still maintaining structural integrity (Fig. 3. 3D). The interstitial cells exhibited very clear morphology and contained large numbers of oleosomes (Fig. 3. 4). While the infected and interstitial cells contained oleosomes as well as starch grains, the

cells where bacteroids have degenerated did not. Oleosomes were abundant in vascular tissue especially in the vascular parenchyma (Fig. 3. 5A). An electron microscopic view of the oleosomes show electron translucent areas (Fig. 3. 5B). The periderm tissue showed considerable autofluorescence (Fig. 3. 5C) and the cell walls appeared positive for the sudan III test indicating the presence of suberin. Some of the cells of the exodermis showed a lack of thickening in semi-thin sections stained *en bloc* with p-phenylenediamine (Fig. 3. 5D).

The most striking change in the winter nodules was the increase in the oleosome population (Table 3. 1) and concomitant increase in the total lipid content (Fig. 3. 6). In all the tissues, the number of oleosomes was significantly higher in winter nodules than in summer nodules. Even the senescent zone contained higher numbers of oleosomes in winter. The vascular tissue and invasion zone showed significantly higher numbers of oleosomes in winter and summer nodules, respectively.

3. 5. Discussion

Overwintering of perennial nodules is a common phenomenon in perennial legumes of temperate regions (Pate, 1961; Bergersen et al., 1963). Bulges on the beach pea nodules indicate the regrowth from the previous season after being dormant over the winter (Bal and Khetmalas, 1996). The presence of a large pink (leghemoglobin containing) symbiotic zone and a short senescent zone in mature nodules indicates nitrogen fixation activity supporting vigorous growth of plants in the summer. The large senescent zone and lack of pink colouration in winter nodules reflect the absence of

nitrogen fixation. Moreover, almost all nodule tissues are dormant in winter except perhaps the vascular tissue. Bergersen et al. (1963) reported the persistence of vascular tissue in the basal senescent portion of overwintering clover nodules under alpine conditions. In the present investigation, well-preserved vascular elements were observed in winter, indicating their functional integrity and connection with the vascular system of the roots. The survival of bacteria, without any loss of structural morphology, in the persistent infection thread during winter is surprising. The mechanism of survival is not clear at present. It may result from the protection by an extracellular matrix that surrounds the bacteria.

Organisms usually store food reserves before a forthcoming dormant period or during an unfavourable environmental condition. Lipids are a more efficient and convenient form of energy storage than starch or proteins (Huang, 1992; Murphy, 1993). Bal and Khetmalas (1996) have pointed out that oleosomes may play an important role during winter dormancy of nodules. In the present study, oleosomes are localized in all nodule tissues from both winter and summer. A higher number of oleosomes in the vascular tissue of winter nodules probably serves to meet the high-energy demand for protection against winter by keeping the vascular supply operative. In summer nodules, large numbers of oleosomes are found in the invasion zone and the meristem. Oleosomes in the invasion zone most likely serve the demands for high-energy requirement during proliferation of membranes and multiplication of bacteroids. In the apical meristem, energy demand for cell division and enlargement has to be satisfied.

Nodule oleosomes have been considered transient storage organelles (Jayaram and Bal, 1991) that are utilized as a supplementary source of carbon and energy for nitrogen fixation in peanut nodules (Siddique and Bal, 1992). On the other hand, it has been mentioned that oleosomes in perennial root nodules may be involved in the dormancy of the nodule tissue during over-wintering (Bal and Martin, 1997; Martin et al., 1998a). Significantly higher numbers of oleosomes in histological preparations from winter nodule tissue compared with summer nodule tissue in beach pea may indicate the participation of lipids in protection from cold stress (Somerville and Browse, 1991). This may be achieved by the antifreeze properties of lipids and their derivatives such as glycerol and fatty acids during low temperature stress. In some animals, triglycerols stored under the skin (Lehninger et al., 1993) and in white adipose tissue (Huang, 1992) serve not only as energy stores, but also as heat insulation against very low temperatures. As winter approaches, the nodules accumulate more lipids in root nodules until early winter. Then nodules start to catabolize the stored lipids as is shown in figure 3. 6.

Lipid catabolism is mainly carried out by β -oxidation, which leads to complete degradation of fatty acids (Gerhardt, 1992). In addition to insulation, the energy released during β -oxidation may also be used for membrane transport and synthesis of precursors for low metabolic activities. The reduction in the rate of metabolism has been reported in several hibernating mammals during winter (Aloia, 1988). Along with energy, oxidation of lipids also yields water which may be required for metabolic activity. Lipolytic activity has been reported in nodule oleosomes of peanut (Jayaram and Bal, 1991). In the present investigation, the electron transparent areas in oleosomes of winter nodules most likely

indicate lipolysis. This is further indicated from the close association of oleosomes with mitochondria, which are involved in fatty acid catabolism. Therefore, the function of oleosomes may be considered as multipurpose: to release energy for metabolism, to prevent freezing in the localized microenvironment of the cells, to insulate cells and supply fatty acids for membrane synthesis during growth.

The cell walls of the outer layers of the cortex in perennial nodules have been shown to be suberized in all parts of the nodule (Pate, 1961). In the present study, autofluorescence of vascular tissue and peridermal cells indicate the deposition of suberin, which could act as a barrier to pathogen entry, and gas and water diffusion (Smith et al., 1986; Jacobsen et al., 1998). However, localized non-suberized cell walls of some cells possibly regulate the gas/water diffusion pathway.

Table 3. 1. Distribution of oleosomes in different nodule tissues of beach pea in summer and winter.

Nodule tissue	Number of oleosomes ($\times 10^{-3} \mu\text{m}^{-2}$ of 1.5 μm semi-thin sections)	
	Summer nodule	Winter nodule
Meristem	5.97 \pm 2.68 ^{ab}	29.22 \pm 2.08 ^b
Invasion zone	8.24 \pm 0.52 ^a	21.92 \pm 1.16 ^c
Symbiotic zone (interstitial cells only)	3.80 \pm 0.32 ^{bc}	*
Senescent zone (interstitial cells only)	2.79 \pm 0.24 ^{bc}	33.00 \pm 2.48 ^b
Vascular tissue (vascular parenchyma)	1.91 \pm 0.09 ^c	53.88 \pm 2.51 ^a
Cortical cells (nodule parenchyma)	3.13 \pm 0.16 ^{bc}	33.98 \pm 1.73 ^b

*No oleosome count was done due to the absence of the symbiotic zone in winter.

Values are means (\pm SE) for 10 replications.

Means (\pm SE) followed by different letters in the same column are significantly different at $P = 0.05$ using Duncan's multiple comparison test.

Fig. 3. 1. Photomicrographs showing distribution of oleosomes in different nodule tissues of beach pea. A. Meristem (m, long arrow) and invasion zone (iz). B. Symbiotic zone (sm) with infected cells (if) and uninfected interstitial cells (itu), and cortical cells (ic = inner cortical cells; oc = outer cortical cells). C. Senescent zone. Oleosomes are indicated by short arrows.

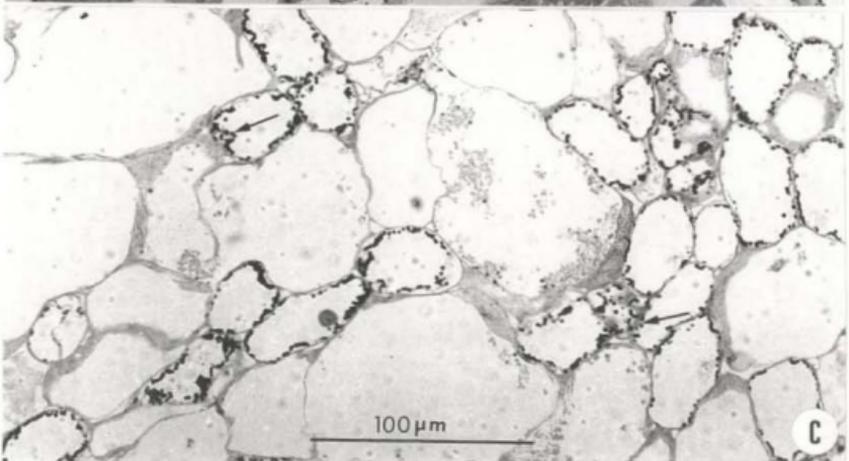
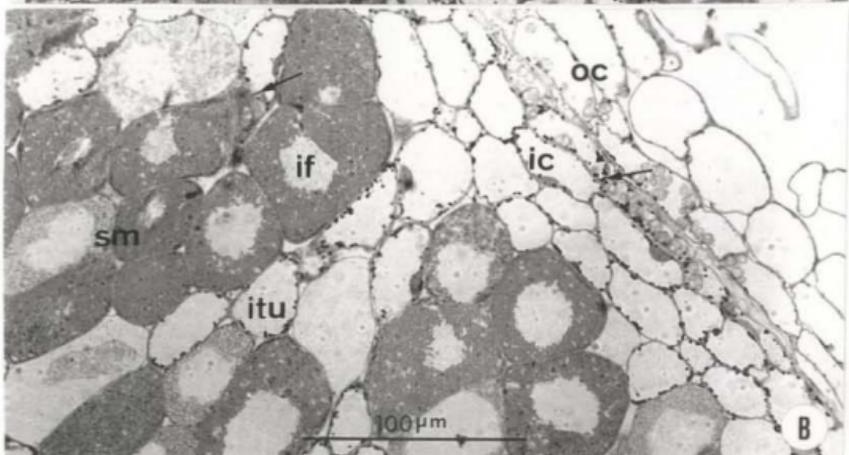
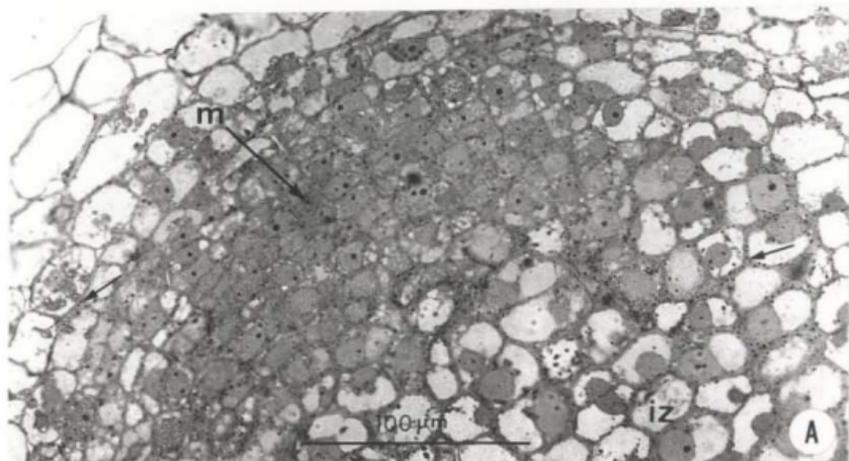
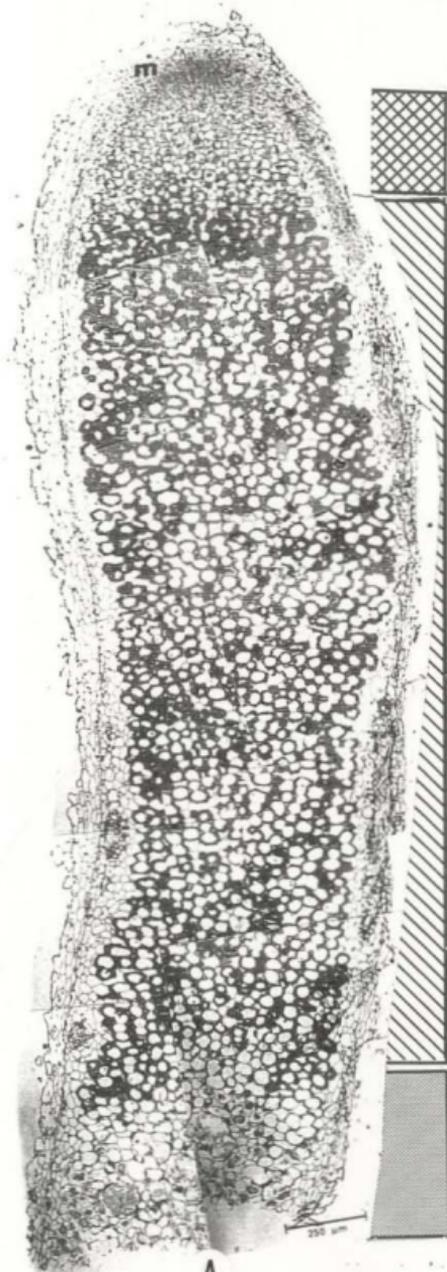
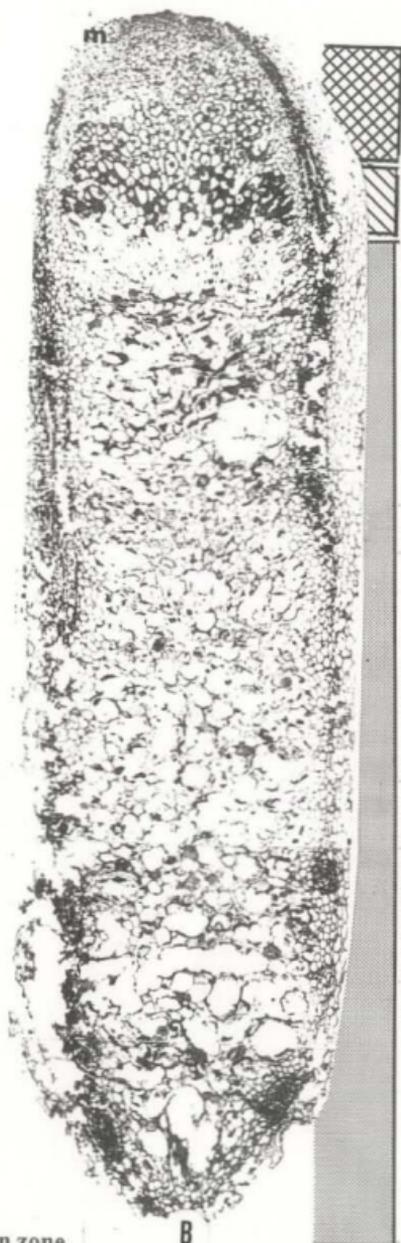


Fig. 3. 2. Montage of photomicrographs showing meristem (m), invasion zone, symbiotic zone and senescent zone in indeterminate nodules of beach pea. A. Mature nodule in summer (July). Note the length of the symbiotic zone. B. Regenerating nodule in spring (April). Note the length of the senescent zone of the previous winter and the newly-formed symbiotic zone.



A



B

-  Invasion zone
-  Symbiotic zone
-  Senescent zone

Fig. 3. 3. Photomicrographs showing changes in winter nodules. A. Photomicrograph showing complete lack of symbiotic zone (now senescent), dormant meristem (dm), dormant invasion zone (div) and senescent zone (sn). B. Electronmicrograph of a meristematic cell showing oleosomes (arrow), nucleus (n) and amyloplasts (a) with starch. C. Electronmicrograph through infection zone showing persistent infection thread (it), oleosomes (arrow) and amyloplasts (a). D. Electronmicrograph of persistent infection thread showing bacteria (b) and extracellular matrix (arrow).

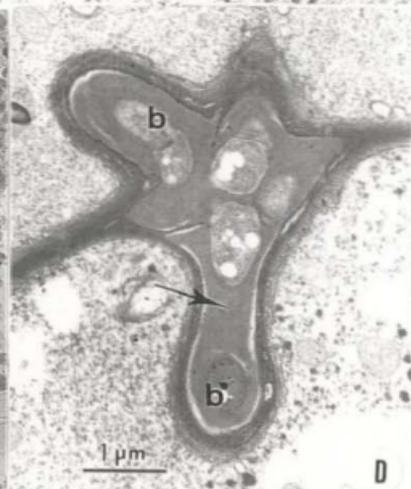
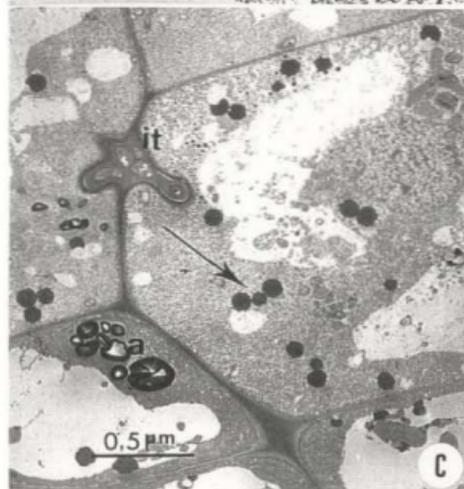
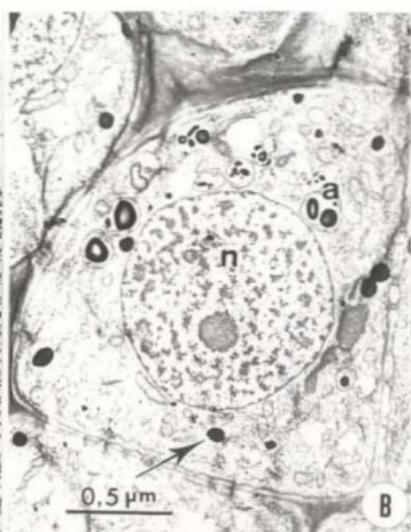
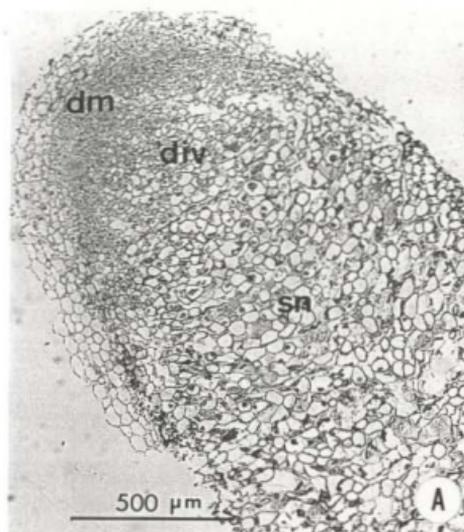


Fig. 3. 4. Photomicrograph showing junction between the invasion zone and the early symbiotic zone in a winter nodule. Note the presence of oleosomes (arrows) and starch grains (s) in the infected and interstitial cells (ic) showing well-preserved morphology of the nucleus (n). The cells containing degenerating bacteroids (db), lack oleosomes and starch grains.



Fig. 3. 5. Photomicrographs of beach pea nodules showing oleosomes and deposition of suberin. A. Photomicrograph of a longitudinal section showing the vascular tissue. Note the vascular parenchyma cells (vp) contain numerous oleosomes (arrows). The xylem (x) and phloem (p) are clearly distinguishable. B. Electronmicrograph of oleosomes (arrow) showing electron transparent areas (☆) are indications of possible lipolysis in winter nodules. Note the close association of oleosome with mitochondria (m). C. Photomicrograph of transverse hand-section showing autofluorescence of the periderm (arrow). D. Photomicrograph of a semi-thin section showing vascular tissue (vt), the thickening of peridermal cell walls and unthickened cell walls (arrow) of some cells of the exodermis.

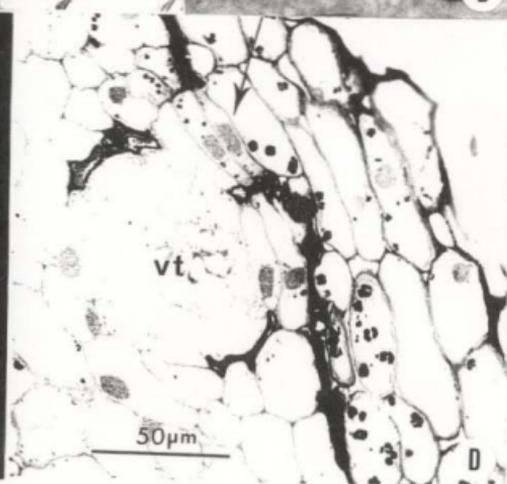
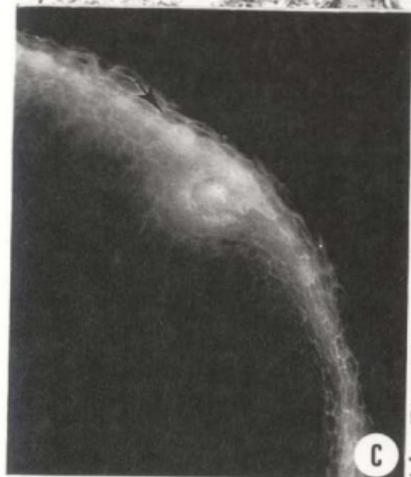
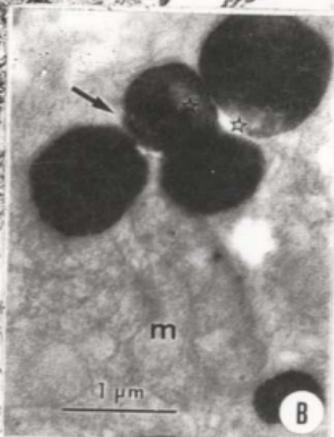
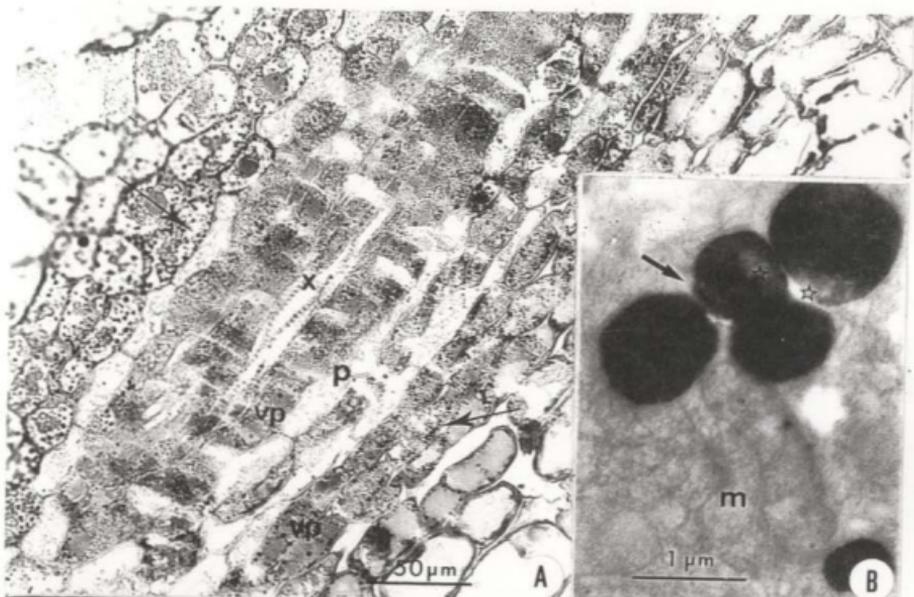
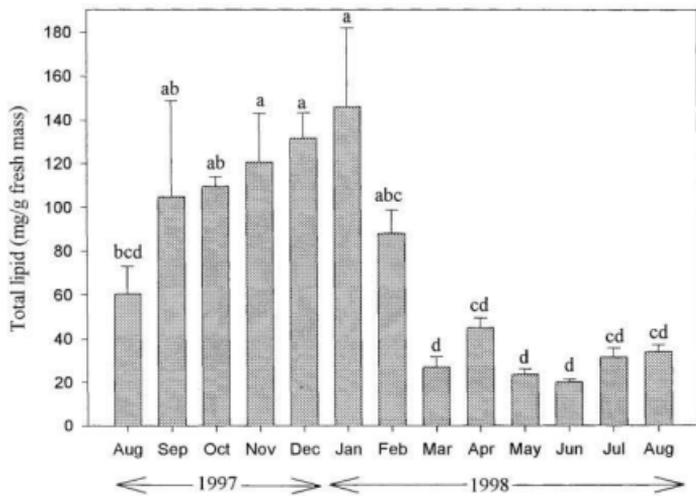


Fig. 3. 6. Seasonal changes in total lipid content of root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar value is the mean of three replications \pm SE.



CHAPTER 4

**EFFECT OF SEASONAL CHANGES ON THE FATTY ACID
PROFILE OF DIFFERENT LIPIDS OF OLEOSOMES ISOLATED
FROM PERENNIAL NODULES OF BEACH PEA**

4. 1. Abstract

Changes in the fatty acid composition of phospholipids (PL), monoglycerides (MG), diglycerides (DG), free fatty acids (FFA) and triglycerides (TG) separated from oleosomes of perennial root nodules of beach pea were analyzed every month for one year. The thin layer chromatography (TLC) analysis revealed that PL and MG are major lipids in nodule oleosomes. The dominant fatty acids are C16:0 followed by C18:0 and C18:1. The fatty acid profile and overall double bond index (DBI) varied among lipid classes depending upon the season. High DBI in PL and MG during the late winter and early spring indicates that they may play a major role in winter survival and regeneration of perennial nodules. The results showed that possible low metabolic activities are taking place in dormant nodules during winter. The levels of many unsaturated fatty acids increased at the expense of saturated fatty acids in almost all lipid classes during winter, which are important to protect the nodule cells from cold stress. Nodules kept some fatty acids and selectively utilized specific fatty acids to survive the winter and subsequent regeneration in spring.

4. 2. Introduction

Lipid content and lipid composition of higher plants vary with environmental conditions and such variation may have an adaptive value (Kuiper, 1985). Lipids are synthesized in plastids and stored in special structures called oleosomes that have been found to occur in the nitrogen-fixing root nodules of many plants (Bal et al., 1989; Barimah-Asare and Bal, 1994; Denduluri and Bal, 1996). The presence of oleosomes in nodules is thought to have an added advantage when β -oxidation is operative along with lipolytic activity, so that the large amounts of energy stored in the oleosomes are mobilized for winter survival (Bal and Khetmalas, 1996), nitrogen fixation and other metabolic functions (Bal and Siddique, 1991; Siddique and Bal, 1991, 1992).

In the present investigation, an attempt was made to separate major oleosomic lipids from perennial root nodules of beach pea collected monthly for one year. The isolated lipids were used for the analysis of fatty acid composition.

4. 3. Materials and Methods

4. 3. 1. Plant materials and sampling

Perennial nodules of beach pea were collected as described in chapter 3 (see section 3. 3. 1 on page number 42).

4. 3. 2. Isolation of oleosomes

Nodules of 5 g (fresh mass) each from different seasons were homogenized separately in 15 ml of 50 mM tris-HCl buffer containing 0.5 M NaCl, pH 7.2 (Yatsu and

Jacks, 1972) under cold conditions (4 °C). The contents were centrifuged at 400 g for 5 minutes and the supernatant was again centrifuged at 30,000 g for 20 minutes. The tiny fat pad floating on the top of the supernatant containing the oleosomes (Bal et al., 1989; Jayaram and Bal, 1991; Denduluri and Bal, 1996) was removed and used for the separation of different lipid classes.

4. 3. 3. Separation of oleosomic lipids by thin layer chromatography

Lipids were extracted from the oleosome fraction by using a solvent mixture of chloroform : methanol (1:1 v/v) to separate different lipid classes (Bligh and Dyer, 1959). A few crystals of hydroquinone were added as an antioxidant to the solvent mixture. Out of two layers formed, the top layer was removed by aspiration and discarded. The chloroform in the bottom layer was evaporated under nitrogen gas, then 25 µl of chloroform was added and mixed well. The samples, each contained 2 mg of oleosomes, were chromatographed on Silica Gel TLC plates (Fisher Scientific, Toronto, Ontario, Canada) along with lipid standards for PL, MG, DG, FFA and TG. The TLC plate was developed in a mixture of hexane/diethyl ether/acetic acid (85:15:2 v/v/v) for the separation of different lipid classes. After brief air-drying, the plate was allowed to react with iodine in a closed chamber. The lipid spots stained a yellow colour were collected after identifying with standards and used for fatty acid analysis. For the photographic purpose, some chromatograms were sprayed with 70% H₂SO₄ and lipids were visualised by charring at 110 °C for 1 hour.

4. 3. 4. Analysis of fatty acid composition

The lipids of oleosomic fractions separated by TLC were used to determine the fatty acid composition using gas chromatography (Hewlett-Packard Series II, Type 5890, Hewlett-Packard Mississauga, Ontario, Canada) (Denduluri and Bal, 1996). The samples were transmethylated by the addition of 2 ml of a 6% mixture containing H_2SO_4 and methanol (1: 15 v/v) with a few crystals of hydroquinone, and heated at 70 °C in an oven (Blue M Electric Co., Illinois, USA) for 5 hours. Then the methylesters were extracted three times with 2 ml of hexane. The hexane layers were combined and rinsed three times with distilled water to remove residual H_2SO_4 . The hexane was then evaporated under nitrogen gas in a fumehood. The residual samples were dissolved in 20 μ l of carbon disulphide and 0.25 μ l was used for gas chromatographic analysis. Fatty acids were separated using a gas chromatograph having a Supelco wax10 column (Supelco, Mississauga, Ontario, Canada), a flame ionization detector and a split/splitless injector in split mode (1:50 split). The detector and injector temperatures were 240 °C and the oven temperature was 200 °C. Total run time was 40 minutes. Helium was used as a carrier gas. The fatty acid peaks were identified by comparing their relative retention times with those of reference fatty acids. The contents of 10 major fatty acids [C14:0 (myristic acid), C14:1 (myristoleic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (linolenic acid), C18:4 and C20:4 (arachidonic acid)] identified in the samples were calculated from the integration data of the chromatographed fatty acids.

4. 3. 5. Double bond index

The DBI was calculated (Skoczowski et al., 1994) using the formula:

$$\text{Double bond index} = \frac{\sum (\text{percentage of fatty acid content} \times \text{number of double bonds})}{100}$$

4. 3. 6. Statistical analysis

All experiments were replicated three times, and mean values and standard errors reported. For all sets of data, one-way analysis of variance was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test at $P = 0.05$. The data in percentage were transformed to arcsine values for the purpose of statistical analysis (Snedecor and Cochran, 1980; Zar, 1996).

4. 4. Results

4. 4. 1. Oleosomic lipids

Five lipid classes (PL, MG, DG, FFA and TG) of nodule oleosomes from different seasons were separated using TLC (Fig. 4. 1). The stained TLC plate revealed that the PL and MG bands were more intense than DG, FFA and TG bands in all monthly oleosomic fractions. This indicates PL and MG as the major form of lipids in oleosomes of perennial root nodules. Overall, irrespective of season, C16:0, C18:0 and C18:1 were the predominant fatty acids in all lipid classes (Figs 4. 2 – 4. 6).

4. 4. 2. Seasonal changes in fatty acid composition of PL

High amounts of C14:0 and C14:1 were observed in the early fall as well as early winter in nodule oleosomes (Fig. 4. 2). C16:0 showed an increase in the early winter and a decrease at the end of winter. A relatively high quantity of C16:1 was present in the PL of nodule oleosomes during August 1997 and it declined throughout winter and summer. The content of C18:0 increased in fall and then decreased in winter with a major drop of about 10% in January and May. At the end of summer, the level of C18:0 again peaked. Low quantity of C18:1 was detected in fall and winter seasons, while it was high at the end of summer. Unsaturated fatty acids such as C18:2 and C18:3 were significantly high at the transitional stage (April/May) from winter to spring. C18:4 did not show significant seasonal changes. High amount of C20:4 was recorded in November.

4. 4. 3. Seasonal changes in fatty acid composition of MG

High amounts of C14:0 and C14:1 were observed during October and February respectively (Fig. 4. 3). Most of the C16:0 and C18:0 were used at the beginning of growing season (April/May). C16:1 was high during August 1997 and January 1998. C18:1 increased in early summer and decreased in late summer, fall and winter. C18:2 increased from February until May. C18:3 was high in the late spring and early summer, whereas C18:4 and C20:4 found high in nodule oleosomes collected during the mid-winter.

4. 4. 4. Seasonal changes in fatty acid composition of DG

High content of C14:0 was recorded during the mid-fall and the early winter (Fig. 4. 4). Nodules collected during the early winter showed high quantity of C14:1. Large amounts of C16:0 were utilized in June. While the content of C16:1 was high in January, C18:0 was relatively high in August 1998. C18:1 peaked in May, whereas C18:2 content was more in March. Oleosomic DG from mid-winter to late summer nodules showed higher amounts of C18:3. Large quantities of C18:4 and C20:4 were recorded during June and November respectively.

4. 4. 5. Seasonal changes in fatty acid composition of FFA

Nodule oleosomes contained high quantities of C14:0 and C14:1 in March and February, respectively (Fig. 4. 5). The content of C16:0 in oleosomic FFA increased during mid-fall season and decreased during the end of winter. The level of C16:1 decreased throughout fall season. C18:0 was relatively high during December and the end of winter, early spring and late summer. The amount of C18:1 decreased during fall season and increased at the beginning and end of winter. Unsaturated fatty acids such as C18:2, C18:3, C18:4 and C20:4 were high during the mid-winter.

4. 4. 6. Seasonal changes in fatty acid composition of TG

High amounts of C14:0 and C14:1 were found in nodule oleosomic TG collected during February and October respectively (Fig. 4. 6). C16:0 was relatively high during the fall and early winter, and most of this fatty acids were used up in March and May.

Both C16:1 and C20:4 gradually increased from August to November and then decreased. C18:0 was found to increase from mid-winter and peaked in July. The level of C18:1 was high throughout the fall, winter (except February) and spring. Large amounts of C18:2 were recorded in March, whereas nodule oleosomic TG from August 1997 showed high amounts of C18:3 and C18:4.

4. 4. 7. Seasonal changes in DBI of oleosomic lipids

PL of nodule oleosomes showed high DBI value during the spring (April/May) (Fig. 4. 7). In MG, the DBI gradually increased from the beginning of winter and reached a peak in May. The DBI was high in November in DG. The DBI of FFA decreased throughout the fall and again increased from the winter to spring. In TG, high DBI was observed during August 1997.

4. 5. Discussion

Previous studies in Jack bean nodules showed PL as the dominant lipid in total lipid fraction followed by TG, DG, MG and FFA (Lynd and Ansman, 1988). In the present study though the individual lipid classes were not measured, the TLC analysis with the same quantity of oleosomes indicated that PL followed by MG are the major lipids in oleosomes of perennial root nodules in all seasons. This is based on the high intensity of PL and MG bands compared to DG, FFA and TG bands after charring. Higher PL and MG in summer and fall may be related to nodule metabolism such as the proliferation and multiplication of bacteroids. Large amounts of membrane materials are

required to enclose bacteroids, since there is synchronous maturation of thousands of symbiosomes in the same infected cell (Roth and Stacey, 1989). Higher PL and MG in winter and spring may play an important role in winter survival and regeneration of perennial nodules. Oleosomes not only store large quantities of energy within the least amount of space (Huang, 1992) but also allow unsaturated fatty acids to acclimatize and maintain the tissues at the cellular level (Nishida and Murata, 1996) during winter dormancy.

The fatty acid composition of different lipids isolated from nodule oleosomes shows striking quantitative differences depending on the growing season. As pointed out by Cooke and Burden (1990), it may be an environmental adaptation. Overall in the present study, C16:0 and C18:0 were the predominant saturated fatty acids and C18:1 was the predominant unsaturated fatty acid in nodule oleosomes. In peanut nodules, it has been reported that oleosomes contained relatively higher amount of saturated fatty acids than unsaturated fatty acids (Jayaram and Bal, 1991). The advantage of having saturated fatty acid is that the yield of ATP molecules during complete oxidation is higher than unsaturated fatty acids (Lehninger, 1975). Generally the level of saturated fatty acids was high in non-winter seasons such as summer and fall in almost all lipid classes in the present study. As mentioned in peanut nodules (Jayaram and Bal, 1991), therefore, beach pea nodules with oleosomes should have a distinct physiological advantage in the highly energy demanding process of nitrogen fixation during summer and fall (Stam et al., 1987). Fatty acids are not directly taken up by the symbiosomes. Lipid catabolism in the infected cells could generate organic acids such as succinate or malate that are

transported through the PBM and provide a source of energy (Mellor, 1989). Organic acids have been shown to support higher rates of respiration as well as nitrogenase activity in bacteroids (Ramaswamy and Bal, 1986). In contrast, a high quantity of unsaturated fatty acids was found in root nodules of Jack bean (Lynd and Anzman, 1988) and *Sesbania* (Denduluri and Bal, 1996).

The results of present study reveal that seasonal changes take place in the fatty acid composition of oleosomes that vary among different lipid classes. The results also indicate that though perennial nodules are dormant in winter months, metabolic activities are possibly continuing at a slow rate in order to keep the cells alive. It is evident that nodules selectively utilized specific oleosomic fatty acids that varied between lipid classes depending on the season. For example in PL, C18:0 decreased in winter, which may be due to the conversion of this fatty acid into C18:1, C18:2 and C18:3. Skoczowski et al. (1994) have reported that the overall degree of fatty acid desaturation is not so important as the specific fatty acid in cold adaptation of wheat seedlings. Different adaptive mechanisms using the diversity of lipids have been reported in organisms to overcome the challenges imposed by environmental factors. It includes restructuring of lipid molecular species, restructuring of PL head group, isomerization, and changes in acyl chain length, fatty acid unsaturation, branched chain fatty acids, lipid classes and lipid to protein ratio (Harwood et al., 1994; Los and Murata, 1998).

In the present study, the level of saturated fatty acids decreased in most of the lipid classes during winter but the quantity of most unsaturated fatty acids increased in many oleosomic lipids. In addition to *de novo* fatty acid synthesis, this may be due to

desaturation and elongation of pre-formed acyl chains. The activity of many fatty acid desaturases and elongases is controlled by environment and gene expression (Harwood, 1996; Los and Murata, 1998). In the present study, the DBI did not increase in all lipids during winter. In PL and MG, the DBI was high at the end of winter and the beginning of growing season. This indicates that the PL and MG of nodule oleosomes play a major role in the protection of nodule cells from cold during winter dormancy. As pointed out by Hernandez and Cooke (1996), the relatively large amounts of unsaturated fatty acids in PL may result in a more fluid membrane which can maintain the normal membrane functions in winter. In the growing season, the energy released from the catabolism of oleosomic PL and MG may be involved in the regeneration of nodules and new plant growth. The DBI of DG was high at the end of the fall season. It may be to protect the nodule cells from occasional frost damage before winter. In FFA and TG, the DBI was high during the period of active nitrogen fixation (August 1997). It may be connected to symbiosis. An increase in membrane fluidity has been reported as an important factor for symbiosome development in soybean nodules (Miller and Tremblay, 1983).

The measurements in the present study are expressed in relative weight percentage. Thus, a change in one fatty acid can alter the composition of other fatty acids. Since PL and MG are major lipids in the oleosomic fractions, changes in other lipids are likely to be of less significance.

Fig. 4. 1. Thin layer chromatography of oleosomes isolated from perennial root nodules of beach pea. The nodules were collected every month from August 1997 to August 1998. Note the separation of phospholipids (PL), monoglycerides (MG), diglycerides (DG), free fatty acids (FFA) and triglycerides (TG) from nodule oleosomes. The intensity of bands indicates PL and MG as the major lipids in nodule oleosomes compared to DG, FFA and TG. The chromatogram was sprayed with 70% H₂SO₄ and lipids were visualised by charring at 110 °C for 1 hour.

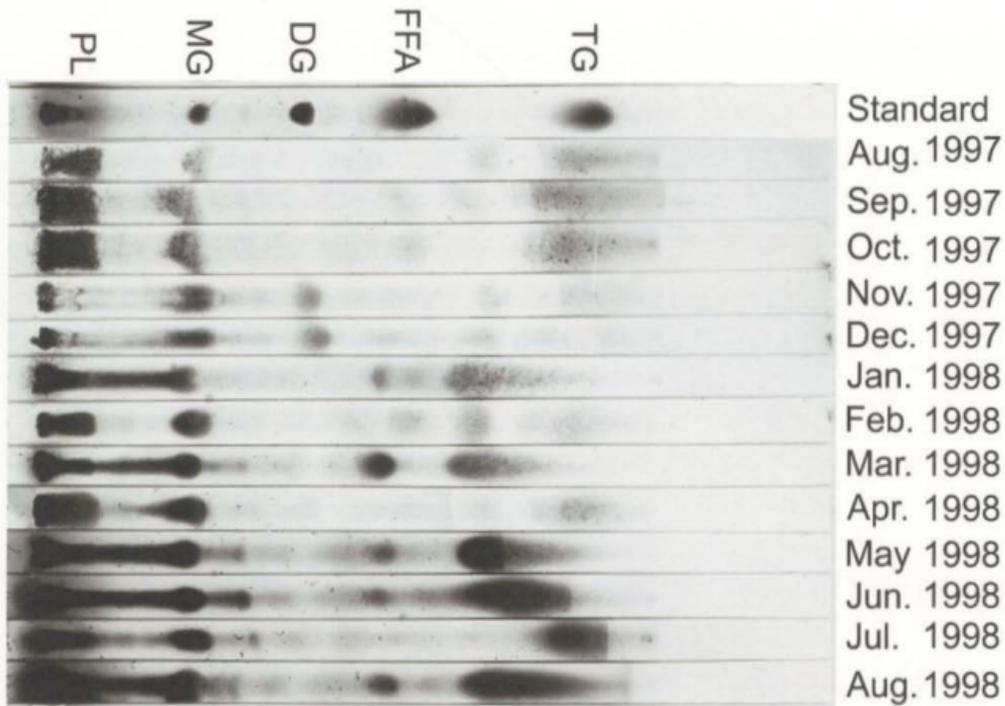


Fig. 4. 2. Seasonal changes in the major oleosomic fatty acid composition of phospholipids (PL) isolated from perennial root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar is the mean (\pm SE) of three replications.

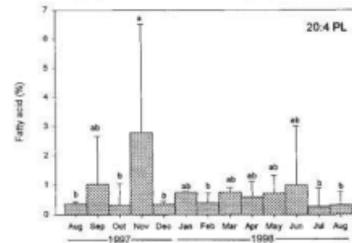
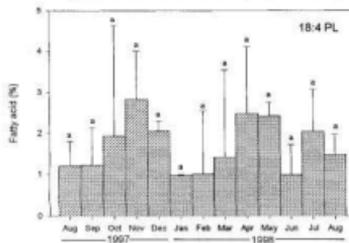
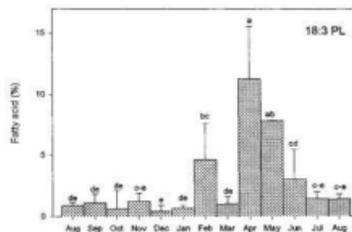
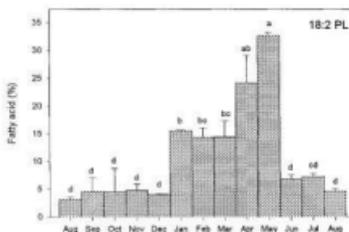
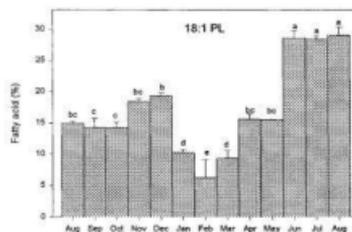
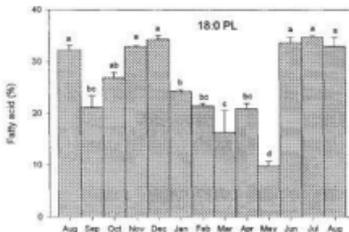
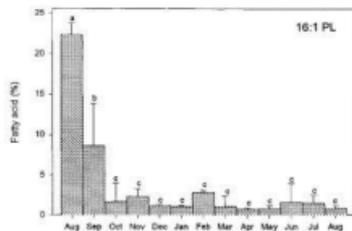
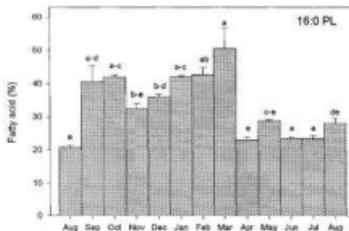
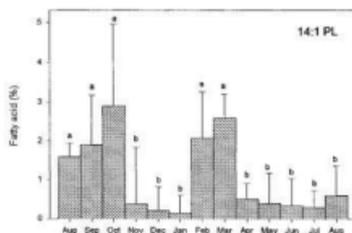
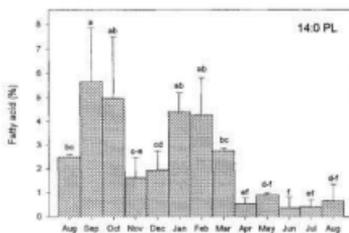


Fig. 4. 3. Seasonal changes in the major oleosomic fatty acid composition of monoglycerides (MG) isolated from perennial root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar is the mean (\pm SE) of three replications.

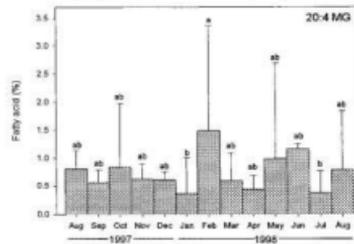
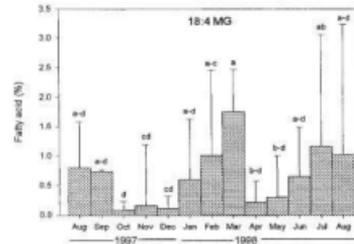
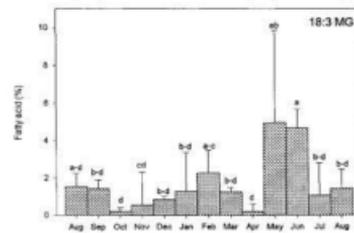
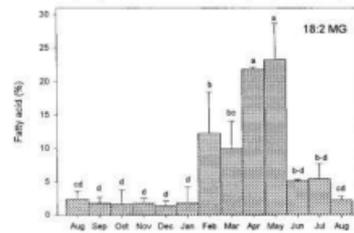
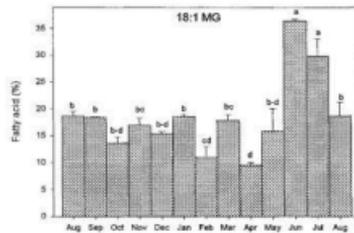
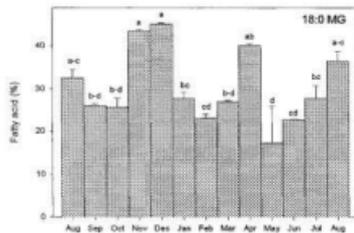
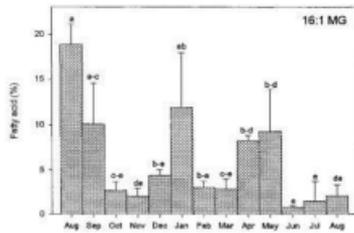
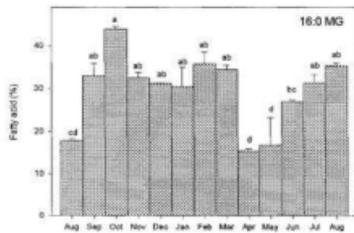
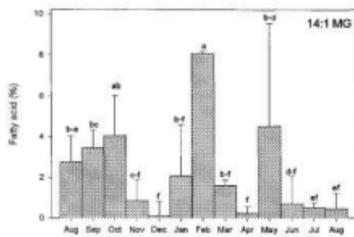
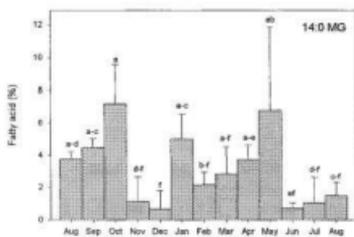


Fig. 4. 4. Seasonal changes in the major oleosomic fatty acid composition of diglycerides (DG) isolated from perennial root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar is the mean (\pm SE) of three replications.

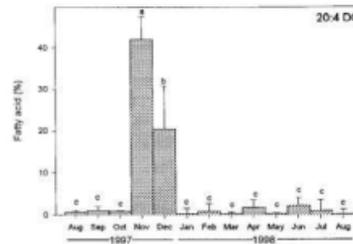
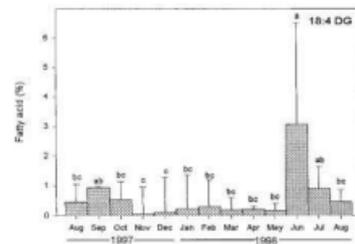
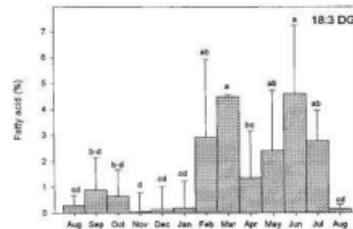
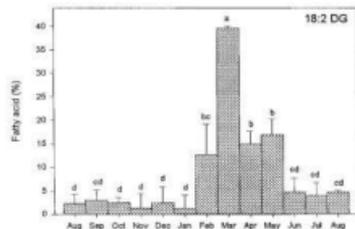
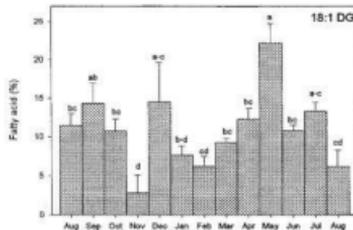
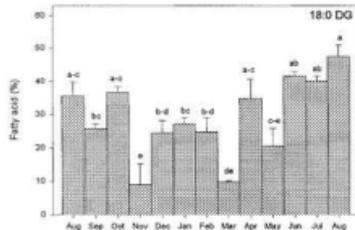
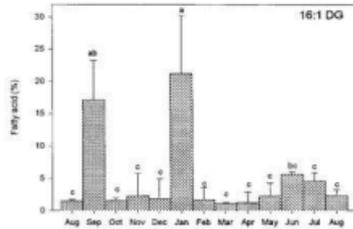
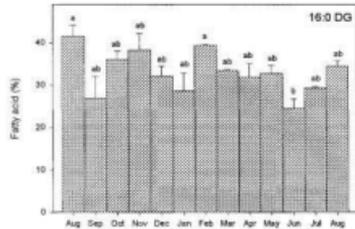
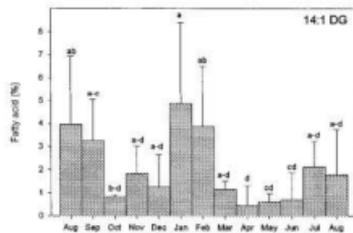
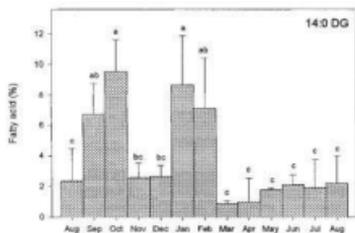


Fig. 4. 5. Seasonal changes in the major oleosomic fatty acid composition of free fatty acids (FFA) isolated from perennial root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar is the mean (\pm SE) of three replications.

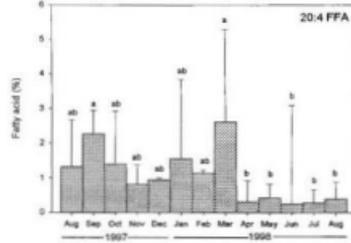
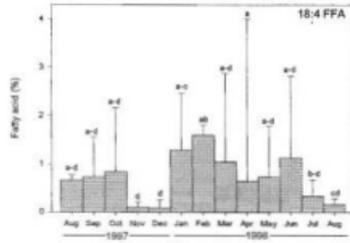
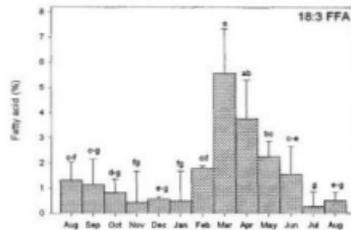
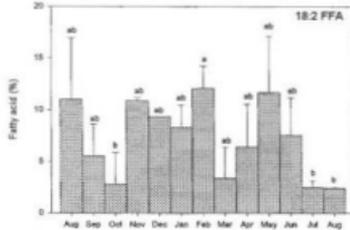
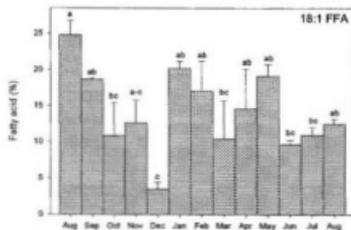
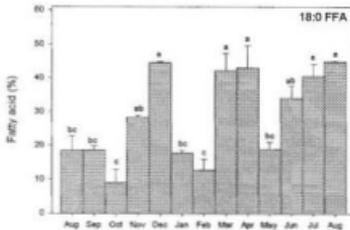
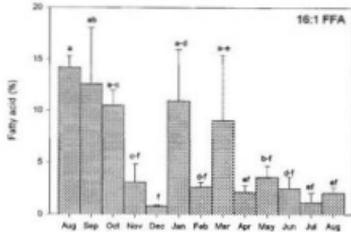
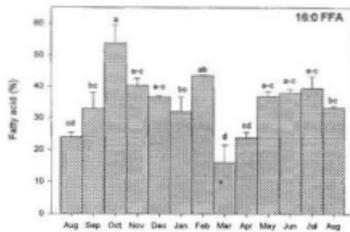
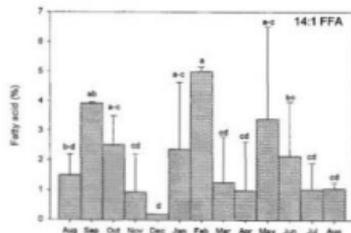
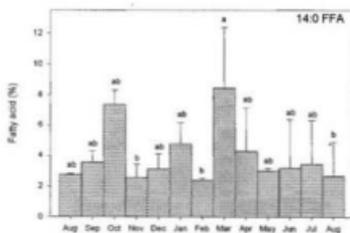


Fig. 4. 6. Seasonal changes in the major oleosomic fatty acid composition of triglycerides (TG) isolated from perennial root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar is the mean (\pm SE) of three replications.

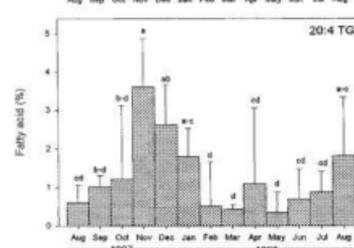
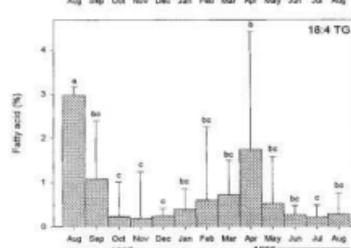
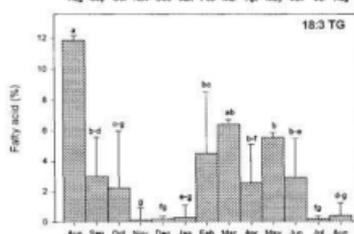
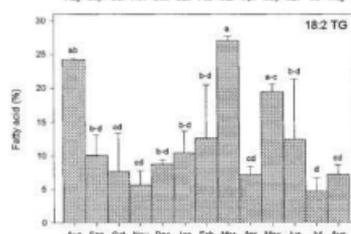
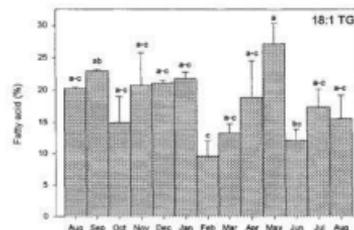
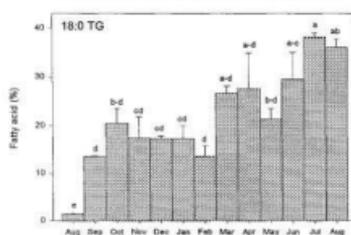
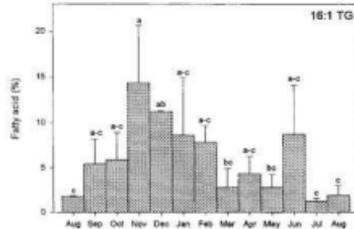
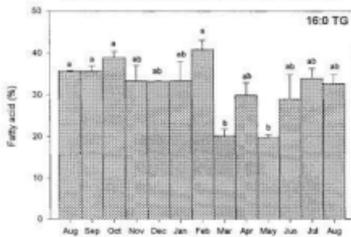
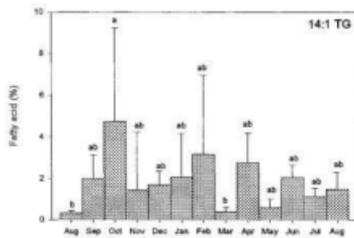
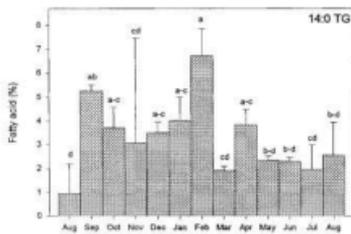
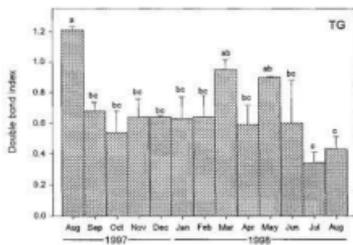
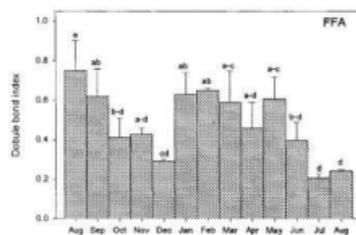
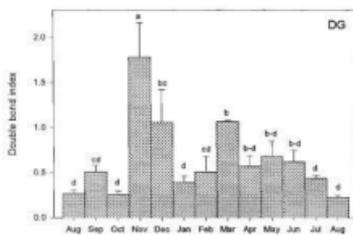
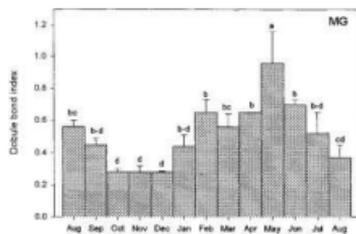
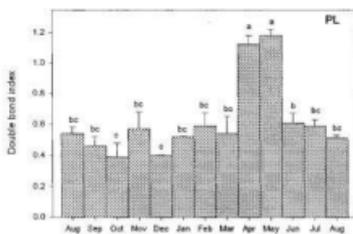


Fig. 4. 7. Seasonal changes in the double bond index of different oleosomic lipids isolated from perennial root nodules of beach pea. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar is the mean (\pm SE) of three replications. PL = phospholipids, MG = monoglycerides, DG = diglycerides, FFA = free fatty acids, TG = triglycerides.



CHAPTER 5

SEASONAL CHANGES IN CARBOHYDRATES OF PERENNIAL NODULES OF BEACH PEA

5. 1. Abstract

Ultrastructural changes in nodule cells of beach pea indicated an accumulation of large amounts of amyloplasts with multiple starch grains in summer months. As the winter season sets in, degradation of amyloplasts and starch grains was detected. The membranes of amyloplasts faded out in winter and the structure of the amyloplasts was lost. The degradation of starch grains showed some electron-dense fiber-like material and amorphous structures. Quantitative data revealed an increase in starch reserves during the summer and depletion during the winter. Total soluble sugar and non-reducing sugar concentrations peaked in the middle of the winter, whereas reducing sugar concentrations showed an increase in the fall. These results indicate that elevated levels of various sugars most likely help to maintain high osmolarity of cells so that the dormant nodules do not freeze during the prolonged periods of cold in winter.

5. 2. Introduction

It is generally believed that current photosynthate (Bach et al., 1958; Dabas et al., 1988) is one of the factors that supports the high-energy requiring process of nitrogen fixation in the symbiosomes within nodules. The electrons for the reduction process and

the carbon skeleton for the incorporation of fixed nitrogen into combined form are also dependent on carbohydrate metabolism (Dilworth and Glenn, 1984). Large amounts of starch in amyloplasts can be seen in nodule cells. In the infected cells, the amyloplasts remain in early stages of development but disappear with the maturation of the bacteroids, when nitrogen fixation and symbiosis begin (Barimah-Asare and Bal, 1994). There is no experimental evidence for direct utilization of starch in nitrogen fixation *per se* (Swaraj et al., 1988), however large quantities of amyloplasts accumulate in the uninfected parenchyma and interstitial cells of symbiotic nodules (Bal and Khetmalas, 1996; Martin et al., 1998a). This present investigation was carried out to determine if starch and other carbohydrates play any role in cold tolerance and winter dormancy of perennial root nodules in beach pea.

5. 3. Materials and Methods

5. 3. 1. Plant materials and sampling

Perennial nodules of beach pea were collected as described in chapter 3 (see section 3. 3. 1 on page number 42).

5. 3. 2. Transmission electron microscopy

Transmission electron microscopy was done as described in chapter 3 (see section 3. 3. 2 on page number 43).

5. 3. 3. Extraction of carbohydrates

5. 3. 3. 1. Sugars

The method employed for extraction of both sugars and starch was essentially that of McCready et al. (1950) with minor modifications. Five hundred milligrams of fresh nodules were homogenized and placed in a 50 ml centrifuge tube, to which 20 ml of 80% (v/v) ethanol was added. The tube was tightly closed and kept in a boiling water bath (Model 0167, Lab-Line Instruments Inc., Illinois, USA) for 20 minutes, and subsequently cooled to room temperature. The supernatant was collected in a 100 ml beaker after centrifuging at 10,000 g for 10 minutes. Again 20 ml of 80% (v/v) ethanol was added to the centrifuge tube, stirred, heated, cooled and centrifuged as before, and the supernatant was collected in the same beaker containing the first extract. This washing was repeated two more times for a total of four washings or until a test with anthrone reagent gave a negative result. The combined supernatants were evaporated to 10 ml on a hot plate and then made up to 50 ml with distilled water and used for the analysis of sugars.

5. 3. 3. 2. Starch

To the residue left after sugar extraction, 10 ml of distilled water was added, and while stirring, 13 ml of 52% (v/v) perchloric acid was added. The mixture was kept cold (4 °C) all the time. The contents were stirred for about 5 minutes continuously with a glass rod and occasionally thereafter for 15 minutes. After that, 20 ml of distilled water was added, stirred and the contents were centrifuged at 10,000 g for 10 minutes. The aqueous starch solution was collected in a 100 ml volumetric flask, and 5 ml of distilled

water was added to the residue and cooled in ice water. While stirring, 6.5 ml of 52% (v/v) perchloric acid was added. As before, the contents were stirred for about 5 minutes continuously with a glass rod and occasionally thereafter for 15 minutes. Then, 10 ml of distilled water was added, stirred and centrifuged at 10,000 g for 10 minutes. The aqueous starch solution was collected in a 100 ml volumetric flask containing the first extract. The second extraction procedure was repeated once more and the supernatants were collected. The combined extract was diluted to 100 ml with distilled water, filtered and used for the analysis of starch.

5. 3. 4. Determination of carbohydrates

5. 3. 4. 1. Sugars

5. 3. 4. 1. 1. Reducing sugars

Total soluble and reducing sugars were estimated according to Nelson (1944). Five hundred microlitres of the above sugar solution (Section 5. 3. 3. 1.) were placed in a 25 ml narrow test tube containing 500 μ l distilled water. Then, 1 ml of alkaline reagent [25 parts of Reagent I + 1 part of Reagent II. Reagent I: 25 g of Na_2CO_3 (anhydrous), 25g of Rochelle salt or sodium potassium tartrate ($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4\text{H}_2\text{O}$), 20g of NaHCO_3 and 200 g of Na_2SO_4 (anhydrous) were dissolved in about 800 ml of water and diluted to 1 litre. Reagent II: 15% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ containing 1 or 2 drops of concentrated H_2SO_4 per 100 ml] was added to the tube. The contents were mixed and heated for 20 minutes in a boiling water bath. Then, the tubes with the solutions were cooled and 1 ml of arsenomolybdate reagent was added (The arsenomolybdate reagent was prepared as

follows: 25 mg of ammonium molybdate was dissolved in 450 ml of distilled water. To this, 21 ml of concentrated H_2SO_4 was added and mixed well. To this solution, 3 g of $Na_2HAsO_4 \cdot 7H_2O$ dissolved in 25 ml of distilled water was added, mixed and placed in an incubator maintained at 37 °C for 24 hours). The contents of the test tube were diluted to 10 ml by adding 7 ml of distilled water, mixed well and the colour intensity was read at 520 nm using a split-beam 1001 spectrophotometer (Bausch and Lomb Inc., New York, USA). Appropriate glucose standards and distilled water were used as blanks.

5. 3. 4. 1. 2. Total soluble sugars

Twenty five millilitres of the above sugar solution (Section 5. 3. 3. 1.) were placed in a 100 ml beaker. To this, 5 ml of HCl:distilled water (1:1 v/v) was added, mixed well and allowed to stand at room temperature for 24 hours for inversion. The sample was then neutralized with 5N NaOH and made up to 50 ml with distilled water. From this diluted sample, 1 ml of aliquot was taken for the estimation of total soluble sugars as mentioned above (Section 5. 3. 4. 1. 1.).

5. 3. 4. 1. 3. Non-reducing sugars

Non-reducing sugars were determined by subtracting the content of reducing sugars from the amount of total soluble sugars (Shahidi et al., 1999).

5. 3. 4. 2. Starch

Five hundred microlitres of sugar-free and filtered starch solution (Section 5. 3. 3. 2.) were diluted with 0.5 ml of distilled water in a 25 x 250 mm borosilicate glass tube. Then, cooled in a water bath and 10 ml of fresh anthrone reagent [0.2% (w/v) in 95% of cold H₂SO₄] was added. The contents were mixed well and heated for 7.5 minutes at 100 °C. The tubes were rapidly cooled to 25 °C in a water bath and the colour intensity of the solution was read at 620 nm using a split-beam 1001 spectrophotometer. A standard curve was prepared using 0 – 100 µg of glucose to estimate the starch content (McCready et al., 1950).

5. 3. 5. Statistical analysis

For all sets of data, one-way analysis of variance was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test at $P = 0.05$.

5. 4. Results

Amyloplasts with multiple starch grains were found to be present in large quantities over the summer months (Fig. 5. 1). The amyloplasts are clearly seen to be surrounded by a membrane. During the winter, profiles of amyloplasts with evidence of degradation became apparent (Fig. 5. 2). The membranes of the organelles appeared faded in electronmicrographs. Morphological appearances of amyloplasts suggested solubilization of starch (Fig. 5. 2A) with intermediate breakdown products, which

appeared fibrillar and amorphous (Fig. 5. 2B). Even lacking a membrane, the outline of the amyloplasts can be clearly distinguished (Fig. 5. 2C and 5. 2D). The starch grains seem to be diminished in size during winter.

A quantitative estimation of starch during the year is shown in figure 5. 3. From August 1997 to August 1998, the measurements reveal a gradual depletion of starch as the winter months approach and then, with the growing season, the loss of the starch reserve is restored. Total soluble sugars (Fig. 5. 4) peak in February and then drop by April/May. Non-reducing sugars also show a similar pattern of increase as that of total soluble sugars during the winter months (Fig. 5. 5). The reducing sugars, on the other hand, revealed a slightly skewed pattern which peaked in October (Fig. 5. 6).

5. 5. Discussion

Carbohydrates are the most abundant biomolecules on earth. They act primarily as stores of chemical energy and as durable building materials for biological structures. A carbohydrate supply is essential for the high-energy demanding process of nitrogen fixation. As well, a carbon skeleton for assimilation and transport of fixed nitrogen is provided by the same metabolic pathway intermediates.

Nodules store carbohydrates in the form of starch (Malek, 1980; Henson et al., 1986), but its role in the nodule physiology is not very clear. Hostak et al. (1987) pointed out that starch metabolism does not occur exclusively in one cell type. Large quantities of amyloplasts can be seen in infected as well as uninfected nodule parenchyma. In the infected cells, the amyloplasts are pushed towards the periphery and, as the symbiosomes

mature, the amyloplasts disappear. Such findings have also been confirmed in both beach pea and alfalfa nodules (Barimah-Asare and Bal, 1994; Martin et al., 1998a). The amyloplasts, however, persist in the uninfected nodule parenchyma. In beach pea nodules, amyloplasts were found to accumulate along with oleosomes before the onset of winter (Bal and Khetmalas, 1996). It has been hypothesized that stored starch may play an important role in its cold tolerance and winter dormancy that lasts for a prolonged period under arctic/subarctic conditions.

In the present investigation, assay of the stored starch and other carbohydrates throughout the year has provided some insight into the possible functioning of carbohydrates in their soluble and stored forms during the dormant periods of the year. The results clearly indicate an increase and build-up of stored starch during the summer months and a dramatic depletion during winter. It is interesting to note that about 40% of the reserve starch is still retained by the end of winter in the perennial nodules, the loss being built up over the next summer. In beach pea, the survey of nodules has revealed regeneration for at least three successive years (Bal, unpublished).

Degradation of amyloplasts has been observed in both fall and winter nodules. The starch grains seem to change revealing some electron-dense fibre-like material as well as some amorphous structures interpreted as degraded intermediates. The membranes fade out in electronmicrographs of winter nodules. The amyloplast structure seems to be lost and smaller remnants of starch grains are apparent. The total soluble sugar and the non-reducing sugar concentrations gradually build up to a peak in the middle of winter, which clearly indicates that the osmolarity of the nodule cells is maintained high so that the

dormant nodules do not freeze. The increase of sugar level is generally correlated with better survival at cold temperatures in a wide variety of plant species (Ristic and Ashworth, 1993; Tronsmo et al., 1993), which has been frequently accompanied by a decrease in the level of starch reserves (Sauter and Cleve, 1991). The graph showing the changes in reducing sugar concentrations (Fig. 5. 6) seemed to be skewed toward the fall season, when cold temperatures set in, with occasional frosts. These reducing sugars are possibly glucose which is the breakdown product of starch. Therefore, these sugars serving as cryoprotectants may be involved in cold tolerance of dormant nodules.

Fig. 5. 1. Electronmicrograph of beach pea nodule collected during August 1997 showing amyloplasts with starch grains (S). Note the clearly visible amyloplast membrane (arrows) around numerous starch grains.

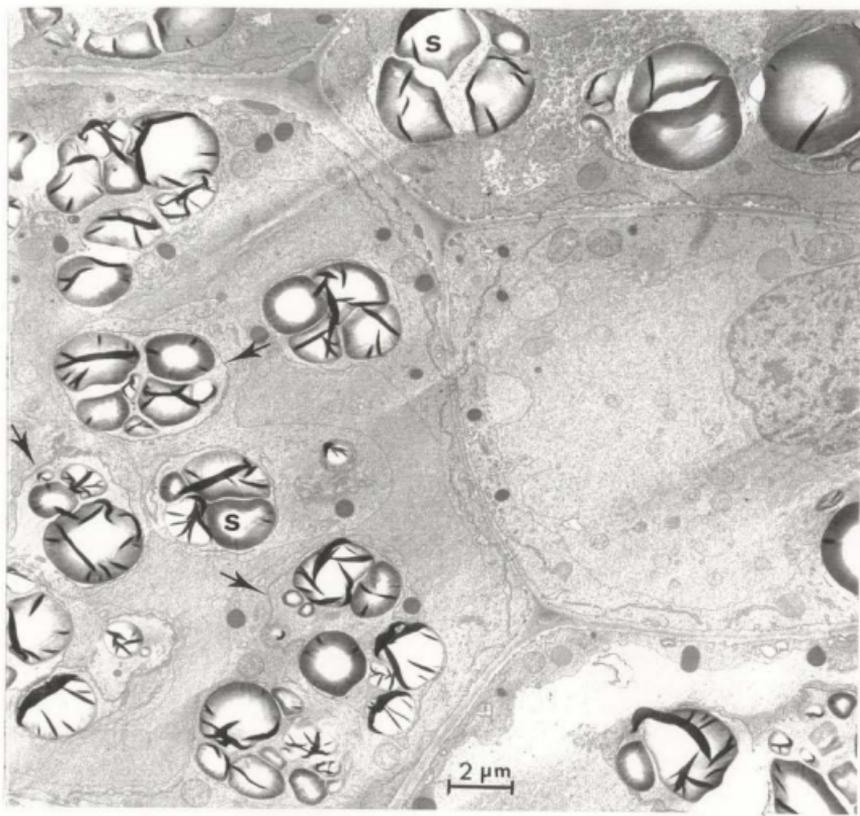


Fig. 5. 2. Electronmicrographs showing the changing patterns in the ultrastructure of amyloplasts of perennial root nodules in beach pea. A. Starch grains in winter months showing ongoing degradation (arrow). B. Nodule from winter months showing degradation of starch (arrow) and the entire amyloplast (a). C. Electronmicrograph showing amyloplast (a) with small starch grains at the end of April 1998. D. Electronmicrograph showing starch grain with a different pattern of degradation during the end of April 1998. In C and D, part of the cell wall (cw), and in C, a tangential section of the nucleus (n) are visible.

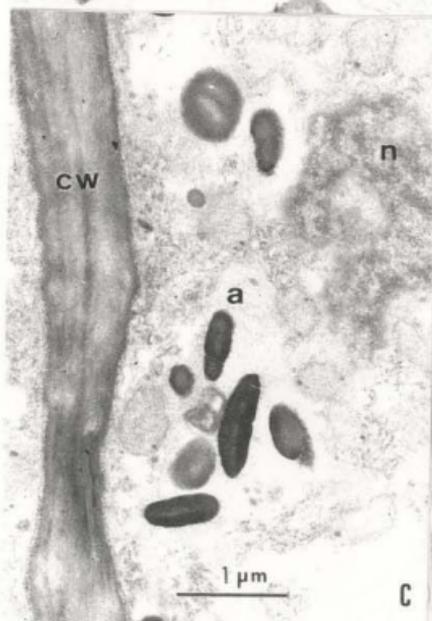


Fig. 5. 3. Seasonal changes in starch content of root nodules of beach pea. Means (\pm SE) were calculated from three replications. Significant differences at $P = 0.05$, indicated by different letters, were calculated using Duncan's multiple comparison test under one-way ANOVA.

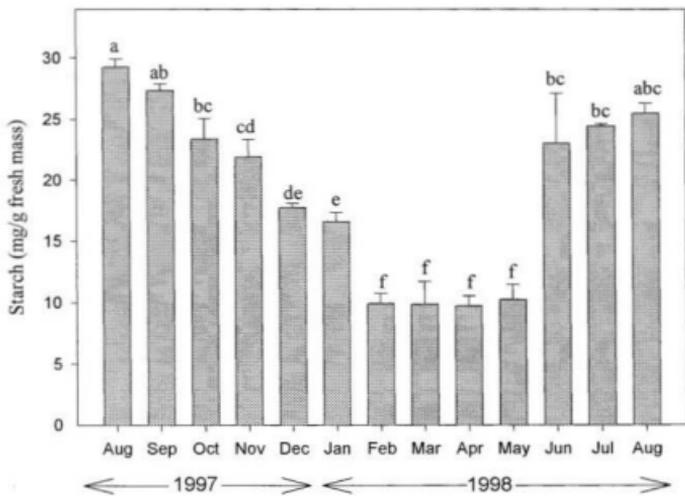


Fig. 5. 4. Seasonal changes in total soluble sugars of root nodules of beach pea. Means (\pm SE) were calculated from three replications. Significant differences at $P = 0.05$, indicated by different letters, were calculated using Duncan's multiple comparison test under one-way ANOVA.

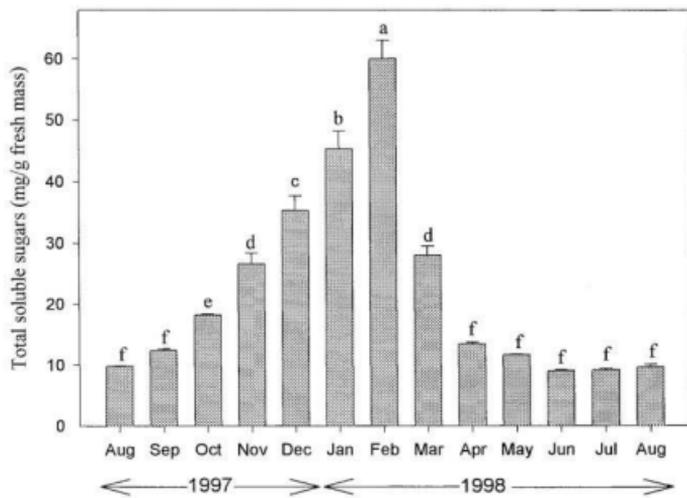


Fig. 5. 5. Seasonal changes in non-reducing sugars of root nodules of beach pea. Means (\pm SE) were calculated from three replications. Significant differences at $P = 0.05$, indicated by different letters, were calculated using Duncan's multiple comparison test under one-way ANOVA.

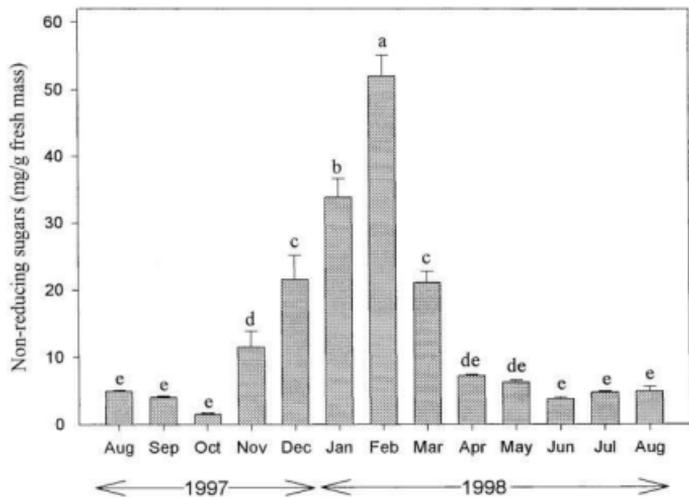
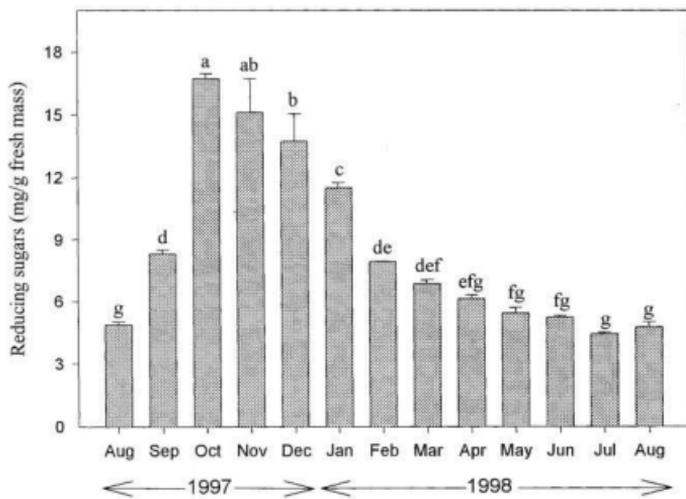


Fig. 5. 6. Seasonal changes in reducing sugars of root nodules of beach pea. Means (\pm SE) were calculated from three replications. Significant differences at $P = 0.05$, indicated by different letters, were calculated using Duncan's multiple comparison test under one-way ANOVA.



CHAPTER 6
SEASONAL CHANGES IN PROTEIN, AMINO ACID AND
ELEMENTAL COMPOSITION OF PERENNIAL NODULES OF
BEACH PEA

6. 1. Abstract

Changes in proteins, amino acids and elements were studied in the perennial nodules of beach pea during winter, summer and fall. Accumulation of total protein content in the nodules increased from mid-summer to early winter and then decreased. Among the total amino acids studied, arginine, cystathionine, ethanolamine, histidine, hydroxyproline, ornithine and proline were found to increase in winter nodules. While γ -aminobutyric acid was found to be significantly higher in fall and summer, sarcosine was higher in summer and winter. Large amounts of K followed by Ca were found in almost all nodule tissues. P, Al, Si and Cu showed significant distribution among different nodule tissues in winter and summer. In the nodular tissue, significantly larger amounts of Na, K and Mg were found in the winter and S in the summer. In both seasons, no significant difference could be observed in the distribution of Cl, Mo, Ca, Mn, Fe and Zn among nodule tissues. Irrespective of nodule tissues, P, K, Ca, Mn, Cu and Zn were found to increase in winter nodules. Summer nodules showed higher amounts of Cl, Na, S, Mg, Al, Si, Mo and Fe. Possible roles of some elements in winter and summer nodules have been suggested.

6. 2. Introduction

Plant cells have been reported to accumulate substantial amounts of proteins (Cloutier, 1987; Kang and Titus, 1987; Mohapatra et al., 1987a,b), proline (Hare et al., 1998, 1999) and other amino acids (Kouchi and Yoneyama, 1984; Fougere et al., 1991) in response to various abiotic stresses. The range of concentrations of a particular element varies widely between different plants and is also affected by the environmental conditions under which the plants are grown (Wytenbach et al., 1985). Elements such as Mo, Fe, P, S and K are essential for nitrogen fixation and various nodule activities (Sprent, 1979). Therefore, analysis of changes in protein, amino acid and elemental composition during different seasons is likely to be useful in understanding the metabolic basis of winter survival strategies in the perennial nodules of beach pea.

6. 3. Materials and Methods

6. 3. 1. Plant materials and sampling

Perennial nodules of beach pea were collected as described in chapter 3 (see section 3. 3. 1 on page number 42). In addition, beach pea nodules collected on 5 September 1998 from Salmon Cove Sandy Beach of Newfoundland were also used.

6. 3. 2. Extraction and estimation of total proteins

After extracting the total lipids as mentioned in chapter 3 (see section 3. 3. 5 on page number 44), the pellets which settled at the bottom of the centrifuge tubes were

collected and dissolved in 5 ml of 1N NaOH. These were used for the determination of total protein content according to the method described by Lowry et al. (1951).

6. 3. 3. Analysis of total amino acid composition

Fresh nodules weighing 25 mg were hydrolyzed in 1 ml of 6N HCl (Carraway and Leeman, 1973; Blackburn, 1978; Ozols, 1990) with 0.05% phenol for 24 hours at 110 °C using 16 × 100 mm culture tubes with teflon-lined screw caps (Gehrke et al., 1985). The tubes were purged for 5 min with nitrogen before capping. The HCl was removed under vacuum and the dried samples were reconstituted using a sodium citrate buffer at pH 2.2 for analyses.

In a separate analysis, tryptophan was determined by hydrolysis of the sample with 1 ml of 3N mercaptoethanesulphonic acid (Penke et al., 1974) for 24 hours at 110 °C in nitrogen-purged Corning culture tubes with teflon-lined screw caps (Gehrke et al., 1985). The mercaptoethanesulphonic acid was neutralized with 3N LiOH and adjusted to pH 2.2 prior to analysis. Cystine and methionine were estimated after performic acid oxidation (Schram et al., 1954; Blackburn, 1968; Ozols, 1990) and prior to hydrolysis in 6N HCl at 110 °C for 24 hours. Cystine and methionine were measured as cysteic acid and methionine sulphone, respectively (Blackburn, 1978).

Using a Beckman Model 121 MB amino acid analyzer (Beckman Instruments Inc., Palo Alto, California, USA) equipped with a cation exchanger resin column (Benson D – x 8.25 bed size 200 x 2.8 mm), the individual amino acids were separated, identified

and measured at the Amino Acid Facility, Department of Biochemistry, Memorial University of Newfoundland, St. John's, Canada.

6. 3. 4. Elemental analysis

Dried nodules were gently split into two halves with a clean razor blade and mounted on aluminum stubs by using two-sided carbon adhesive tapes (J.B. EM Services Inc., Dorval, Quebec, Canada). After coating with carbon using a DV-502 A carbon coater (Denton Vacuum Inc., New Jersey, USA), samples were examined in a Hitachi S570 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) equipped with an X-ray analyzer at 20 kV. The X-ray analysis, by line scan analyses of 14 selected elements (Na, Mg, Al, Si, P, S, Cl, K, Ca, Mn, Fe, Cu, Zn and Mo), was performed with a Tracor Northern 5500 energy dispersive X-ray analyzer equipped with a microtrace silicon X-ray spectrometer (Model 70152) that has a spectral resolution of 145 eV at a detector/sample take-off angle of 30°. The X-ray spectra were accumulated to give a total of 1024 counts. The concentration of elements was calculated as a relative weight percentage using Tracor Northern's software package. The elemental analysis was done in different nodule tissues; namely, meristem, invasion zone, symbiotic zone, senescent zone, cortical cells and vascular tissue. The location of these nodule tissues is shown in figure 6. 1.

6. 3. 5. Statistical analysis

The experiments on total proteins, total amino acids and elemental composition of nodule tissues were replicated at least three or four times and mean values and standard errors reported. To determine the seasonal changes in elemental composition, data from different nodule tissues of each season were combined and used for statistical analysis. For all sets of data, one-way analysis of variance was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test at $P = 0.05$. For the purpose of statistical analysis, data in percentage were transformed to arcsine values (Snedecor and Cochran, 1980; Zar, 1996).

6. 4. Results and Discussion

6. 4. 1. Total protein content

Cold tolerance in temperate biennials and perennials is the phenomenon that enables them to survive winter conditions and resume growth and development in the spring. It involves genetically-programmed and integrated processes (Guy, 1990). Alterations in the proteins of plant tissues have long been associated with cold survival. This includes both a quantitative increase in protein content and alterations in electrophoretic profiles (Graham and Patterson, 1982; Johnson-Flanagan and Singh, 1988; Guy, 1990).

In the present study, total protein content of beach pea nodules showed an increase from mid-summer to January and then exhibited a decreasing trend throughout the winter (Fig. 6. 2). In the literature, two reasons were mentioned for increases in

protein content during adaptation to stress depending on the plant species: increased *de novo* synthesis and decreased utilization of protein as a consequence of low growth rate (Guy and Haskell, 1987; Johnson-Flanagan and Singh, 1988). The products of degraded proteins in winter may be used in various metabolic functions. The change in proteins during cold acclimation have been reported to occur most probably at the transcriptional level (Mohapatra et al., 1987a,b).

6. 4. 2. Total amino acid composition

The composition of total amino acid in perennial nodules of beach pea in winter, summer and fall is shown in Tables 6. 1 and 6. 2. γ -Aminobutyric acid was the most abundant compound followed by aspartic acid + asparagine and glutamic acid + glutamine. A wide variation in the composition of total amino acid of nodules depending on the season was observed. While some amino acids showed significant seasonal changes (Table 6. 1), many did not show significant changes (Table 6. 2) during winter, summer and fall. Significantly higher amounts of arginine, cystathionine, ethanolamine, histidine, hydroxyproline, ornithine and proline accumulated in winter nodules compared to summer and fall season nodules. γ -Aminobutyric acid was the only amino compound found significantly high in both fall and summer, whereas sarcosine was high in summer and winter. Fougere et al. (1991) have reported that salt stress enhanced the total amino acid concentration in nodules and roots of alfalfa. Similar results have also been reported in other legumes subjected to drought stress (Ford, 1984; Venekamp and Koot, 1988).

Various amino acids have been shown to change in organisms during exposure to different environmental conditions (Antoniw and Sprent, 1978; Kouchi and Yoneyama, 1984; Machackova et al., 1989; Chien et al., 1992; Hare et al., 1998, 1999). Among the amino acids, proline accumulation is one of the most common responses to stress (Borsani et al., 1999) as a result of increased biosynthesis (Fougere et al., 1991). According to Bray (1993), this molecule possesses some peculiar properties: being an efficient osmotic compound it can be accumulated in high concentrations without interfering with cell metabolism. Proline accumulation can play an important role in cellular pH control (Venekamp, 1989; Irigoyen et al., 1992). Moreover, it could serve as a nitrogen and carbon source to be used in stress recovery and it can also act as a hydroxyl radical scavenger and protein protector (Chiang and Dandekar, 1995). In soybean nodules, proline has been postulated to play a role in ureide metabolism, in transferring redox potential from the cytosol to the bacteroids, and in serving as carbon and nitrogen sources for these bacteroids (Khol et al., 1988).

High temperature has promoted the release of phosphoserine, phosphoethanolamine, citrulline, threonine, glycine and ammonia from the root systems of legumes (Ofosu-Budu et al., 1992). Higher amounts of arginine, cystathionine, ethanolamine, histidine, hydroxyproline and ornithine in winter nodules are most likely to provide the protection against low temperature. However, the exact mechanism behind these amino acids in cold protection is not clear at present. *Rhizobium meliloti* bacteroids have been shown to contain a very high level of γ -aminobutyric acid, while free-living cells lacked this compound (Miller et al., 1991). In the present work, increased quantities

of γ -aminobutyric acid during summer (July) and fall (September) might be due to more bacteroids in the active nitrogen-fixing nodules. Some bacteria are seen in dormant winter nodules but the population is much less compared to those in summer and fall. This may be the reason for low quantity of γ -aminobutyric acid in winter nodules.

6. 4. 3. Elemental composition

Plant growth requires the incorporation of elements into plant tissues. With the exception of some trace elements, the mineral requirements of nodulated legumes are not appreciably different from those of plants in general (Smith, 1982). The growth of individual plants may be limited by the extent of adaptation to unfavourable mineral environments (Bollard and Butler, 1966). In the present study, the distribution pattern of elements revealed a wide variation between nodule tissues and seasons (Tables 6. 3 – 6. 6). Elements such as P, Al, Si and Cu were significantly present in nodule tissues during both winter and summer (Table 6. 3). P is an essential part of many sugar phosphates involved in photosynthesis, respiration and other metabolic processes, and it is also part of nucleotides and of the PL present in membranes (Evans and Sorger, 1966; Salisbury and Ross, 1986). In the present work, a large amount of P was found in the nodule meristem during both winter and summer. The principal role of P is in energy transduction by participating in the formation of ATP (Glass, 1989), which may be the reason for the high amount of P in the active region of the nodule, the meristem. In winter, the dormant nodule retains P for the regeneration of nodule activity in the following spring and summer.

Most plants contain very little Al (Sutcliffe and Baker, 1976). In the present work, while cortical cells recorded significantly high Al compared to other nodule tissues in winter, the meristem took over this position in summer. The seasonal role of Al in perennial nodules of legumes is not clear at present. However, nodule formation and the expression of *nod* genes by rhizobia can be sensitive to Al (Bottomley, 1992). The interference of Al with P uptake and metabolism in plants has been reported (Bollard and Butler, 1966). Si is a major inorganic constituent of higher plants (Epstein, 1999). In cortical cells, significantly high Si was found in both seasons in the present investigation. As mentioned by Bollard and Butler (1966) and Glass (1989), this may be used to add strength to the cell walls in which it is deposited. The presence of Si has been shown to increase the resistance to water loss and fungal infection (Bollard and Butler, 1966; Glass, 1989).

Cu is present in several enzymes or proteins involved in oxidation and reduction (Salisbury and Ross, 1986). In the present work, while vascular tissue accumulated more Cu in winter, invasion zone registered high Cu in summer. Oxidation of various substances such as lipid supplies the energy required (Lehninger et al., 1993) to keep the vascular supply active in winter nodules. High Cu in nodule tissues may be involved in this type of oxidation. A high amount of Cu in the invasion zone of summer nodules is most likely needed to participate in oxidation and reduction reactions, which may supply the energy for proliferation and multiplication of bacteroids.

Some elements such as Na, K and Mg showed significant distribution among winter nodule tissues, whereas elemental S was found significantly in summer nodule

tissues (Table 6. 4). Na is considered an essential nutrient for plants capable of fixing CO₂ via C₄ organic acids. Mg is an essential constituent of chlorophyll and also associated with many proteins. In plants, S occurs mostly in proteins, specifically in the amino acids, cysteine and methionine. Other essential compounds that contain S are the vitamins, thiamine and biotin, and coenzyme A, a compound essential for respiration and for synthesis and degradation of fatty acids (Evans and Sorger, 1966; Sutcliffe and Baker, 1976; Salisbury and Ross, 1986; Glass, 1989). In the present work, cortical cells and the meristem in winter nodules showed high Na and Mg respectively, whereas meristems in summer nodules showed high S. Higher K was detected in the senescent zone and vascular tissue than in other nodule tissues. As pointed out by Sutcliffe and Baker (1976) in other plants, K is the main constituent of beach pea nodule tissues. K is an activator of many enzymes that are essential for photosynthesis and respiration and it also activates enzymes needed to form starch and proteins. Elements such as Cl, Mo, Ca, Mn, Fe and Zn did not show significant changes in different nodule tissues of both winter and summer nodules (Tables 6. 5 and 6. 6).

Individual plant species and varieties differ markedly in their elemental composition even when they are grown under the same conditions due to genetic reasons and environmental influences (Casler et al., 1987). In the present study, analyses of elements irrespective of nodule tissues revealed that some elements such as P, K, Ca, Mn, Cu and Zn were found in higher amounts in dormant winter nodules (Fig. 6. 3) and other elements such as Cl, Na, S, Mg, Al, Si, Mo and Fe were present in higher amounts in active summer nodules (Fig. 6. 4). The presence of abundant K has been reported to be a

major contributor to the osmotic potential of cells and therefore their turgor pressure (Sutcliffe and Baker, 1976; Salisbury and Ross, 1986). This may help the dormant nodules to protect their tissues against cellular dehydration caused by cold stress.

As reported in other plant parts (Dhindsa et al., 1997), Ca may be involved in protection against cold stress in perennial nodules. Ca has been reported to act as a second messenger in response to external stimuli (Bush, 1995; Dhindsa et al., 1997). It is also important for growth of higher plants and plays a significant role in the ecology of rhizobia (Bollard and Butler, 1966; Bottomley, 1992). High amounts of Mo and Fe in summer nodules indicate the involvement of these elements in active nitrogen fixation. Both Fe and Mo are essential for nitrogen fixation and for nitrate assimilation. They are part of the nitrogen-fixing enzyme, nitrogenase (Salisbury and Ross, 1986; Lodha and Nainawatee, 1993). Fe occurs in the prosthetic group of certain proteins. Ferridoxin is an Fe-containing protein, which acts as an electron carrier in photosynthetic phosphorylation and also in nitrogen fixation. Leghemoglobin, another haem-protein, is found in nitrogen-fixing root nodules (Bergersen, 1963; Sutcliffe and Baker, 1976).

Table 6. 1. Seasonal changes in beach pea root nodules: significantly changing total amino acid ($\mu\text{g}/\text{mg}$ fresh mass).

Total amino acid	Winter (February 20, 1998)	Summer (July 11, 1998)	Fall (September 5, 1998)
Arginine	6.00 ± 0.39^a	4.20 ± 0.39^{ab}	3.84 ± 0.72^b
Cystathionine	0.06 ± 0.01^a	0.05 ± 0.001^{ab}	0.03 ± 0.003^b
Ethanolamine	0.09 ± 0.01^a	0.01 ± 0.00^b	0.02 ± 0.01^b
γ -Aminobutyric acid	17.61 ± 0.97^b	35.60 ± 3.68^a	41.93 ± 1.29^a
Histidine	3.14 ± 0.26^a	2.55 ± 0.12^{ab}	2.07 ± 0.33^b
Hydroxyproline	4.45 ± 0.34^a	3.46 ± 0.03^b	2.50 ± 0.26^c
Ornithine	0.31 ± 0.03^a	0.14 ± 0.006^b	0.12 ± 0.01^b
Proline	7.64 ± 0.70^a	4.21 ± 0.27^b	3.52 ± 0.58^b
Sarcosine	2.82 ± 0.12^a	2.93 ± 0.36^a	1.54 ± 0.40^b

Values are means (\pm SE) of three replications.

Means (\pm SE) followed by different letters in each row are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Table 6. 2. Seasonal changes in beach pea root nodules: non-significantly changing total amino acid and ammonia ($\mu\text{g}/\text{mg}$ fresh mass).

Total amino acid and ammonia	Winter (February 26, 1998)	Summer (July 11, 1998)	Fall (September 5, 1998)
Alanine	4.96 \pm 0.37	5.50 \pm 0.42	4.63 \pm 0.85
α -Aminoadipic acid	0.02 \pm 0.01	0.01 \pm 0.01	0.00 \pm 0.00
Ammonia	1.40 \pm 0.10	1.89 \pm 0.24	1.38 \pm 0.26
Aspartic acid +			
Asparagine	9.59 \pm 0.72	14.40 \pm 1.72	10.72 \pm 1.70
β -Alanine	0.15 \pm 0.08	0.03 \pm 0.03	0.00 \pm 0.00
Citrulline	0.02 \pm 0.01	0.00 \pm 0.00	0.00 \pm 0.00
Cystine	1.05 \pm 0.08	0.96 \pm 0.06	1.02 \pm 0.13
Glutamic acid +			
Glutamine	8.85 \pm 0.60	9.67 \pm 0.71	8.15 \pm 1.43
Glycine	4.40 \pm 0.35	4.23 \pm 0.30	3.60 \pm 0.64
Hydroxylysine	0.02 \pm 0.02	0.00 \pm 0.00	0.00 \pm 0.00
Isoleucine	4.05 \pm 0.26	4.15 \pm 0.36	3.58 \pm 0.70
Leucine	6.24 \pm 0.58	6.77 \pm 0.19	5.91 \pm 0.99
Lysine	6.80 \pm 0.49	6.70 \pm 0.42	5.44 \pm 0.98
Methionine	1.87 \pm 0.11	1.91 \pm 0.08	1.74 \pm 0.24
Phenylalanine	3.99 \pm 0.25	3.98 \pm 0.23	3.44 \pm 0.64
Serine	4.56 \pm 0.33	4.27 \pm 0.20	3.53 \pm 0.56
Threonine	4.32 \pm 0.29	4.09 \pm 0.27	3.48 \pm 0.63
Tryptophan	0.35 \pm 0.07	0.48 \pm 0.05	0.35 \pm 0.08
Tyrosine	3.67 \pm 0.23	3.47 \pm 0.26	3.10 \pm 0.55
Valine	5.24 \pm 0.34	5.20 \pm 0.41	4.37 \pm 0.82

Values are means (\pm SE) of three replications.

Table 6. 3. Elemental composition (relative weight percentage) of different nodule tissues of beach pea: significantly distributed elements in winter and summer (P, Al, Si and Cu).

Nodule tissues	P		Al		Si		Cu	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
Meristem	12.75 ± 2.96 ^a	8.08 ± 0.43 ^a	4.16 ± 2.03 ^{ab}	7.91 ± 1.02 ^a	0.77 ± 2.09 ^b	2.76 ± 1.33 ^{ab}	4.69 ± 2.05 ^{ab}	0.39 ± 1.82 ^b
Invasion zone	7.27 ± 1.56 ^{ab}	7.80 ± 1.96 ^{ab}	2.20 ± 0.76 ^b	3.71 ± 0.49 ^c	0.77 ± 1.50 ^b	2.29 ± 1.69 ^b	1.97 ± 3.32 ^b	4.71 ± 1.47 ^a
Symbiotic zone	*	6.95 ± 0.78 ^{ab}	*	4.44 ± 1.20 ^{bc}	*	2.92 ± 3.81 ^b	*	2.73 ± 3.25 ^{ab}
Senescent zone	6.68 ± 1.38 ^{ab}	4.09 ± 0.61 ^{bc}	2.95 ± 0.96 ^b	6.44 ± 1.85 ^{abc}	0.37 ± 1.44 ^b	1.18 ± 2.01 ^b	7.61 ± 5.28 ^{ab}	4.63 ± 4.47 ^{ab}
Cortical cells	6.94 ± 2.45 ^{ab}	3.07 ± 1.67 ^c	7.98 ± 2.79 ^a	7.41 ± 1.54 ^{ab}	3.58 ± 1.30 ^a	8.33 ± 0.41 ^a	5.70 ± 1.43 ^{ab}	3.12 ± 1.56 ^{ab}
Vascular tissue	3.19 ± 2.25 ^b	5.83 ± 2.13 ^{abc}	5.17 ± 1.15 ^{ab}	7.28 ± 1.03 ^{ab}	0.37 ± 1.76 ^b	4.53 ± 3.73 ^{ab}	10.48 ± 3.78 ^a	1.89 ± 2.33 ^{ab}

*No elemental analysis was done due to the absence of the symbiotic zone in winter. Values are means (± SE) of four replications.

Means (± SE) in the same column followed by different letters are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Table 6. 4. Elemental composition (relative weight percentage) of different nodule tissues of beach pea: significantly distributed elements either in winter (Na, K and Mg) or summer (S).

Nodule tissues	Na		K		Mg		S	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
Meristem	6.35 ± 1.26 ^{ab}	15.50 ± 1.76 ^a	31.41 ± 1.03 ^b	26.42 ± 1.49 ^a	5.54 ± 0.82 ^a	5.29 ± 1.05 ^a	7.54 ± 2.13 ^a	13.98 ± 2.01 ^a
Invasion zone	6.83 ± 4.71 ^{ab}	16.62 ± 0.98 ^a	38.02 ± 1.92 ^{ab}	25.90 ± 1.96 ^a	3.73 ± 0.63 ^{ab}	5.51 ± 1.50 ^a	6.89 ± 2.44 ^a	11.25 ± 1.83 ^{abc}
Symbiotic zone	*	15.53 ± 1.18 ^a	*	20.86 ± 1.40 ^a	*	6.93 ± 1.13 ^a	*	12.75 ± 2.05 ^{ab}
Senescent zone	1.08 ± 2.99 ^b	12.11 ± 1.34 ^a	40.98 ± 1.10 ^a	26.26 ± 1.96 ^a	2.37 ± 1.08 ^{ab}	6.80 ± 2.75 ^a	8.15 ± 2.46 ^a	7.99 ± 0.84 ^{bc}
Cortical cells	10.06 ± 2.10 ^a	14.82 ± 3.12 ^a	32.56 ± 1.17 ^b	20.48 ± 1.22 ^a	3.46 ± 0.87 ^{ab}	4.86 ± 1.03 ^a	6.57 ± 0.15 ^a	6.73 ± 1.11 ^c
Vascular tissue	7.26 ± 5.31 ^{ab}	14.17 ± 3.63 ^a	40.86 ± 1.49 ^a	22.37 ± 3.47 ^a	2.49 ± 3.84 ^b	6.12 ± 1.94 ^a	3.36 ± 3.30 ^a	8.53 ± 1.14 ^{abc}

*No elemental analysis was done due to the absence of the symbiotic zone in winter. Values are means (± SE) of four replications.

Means (± SE) in the same column followed by different letters are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Table 6. 5. Elemental composition (relative weight percentage) of different nodule tissues of beach pea: non-significantly distributed elements in winter and summer (Cl, Mo and Ca).

Nodule tissues	Cl		Mo		Ca	
	Winter	Summer	Winter	Summer	Winter	Summer
Meristem	3.21 ± 0.83	3.11 ± 0.23	1.85 ± 3.95	2.25 ± 4.37	19.11 ± 0.61	11.95 ± 1.43
Invasion zone	3.25 ± 0.66	8.02 ± 2.75	4.34 ± 4.94	1.12 ± 3.06	20.92 ± 1.68	10.63 ± 0.75
Symbiotic zone	*	4.70 ± 1.34	*	7.02 ± 4.55	*	13.48 ± 1.15
Senescent zone	4.37 ± 0.82	4.68 ± 0.35	1.79 ± 3.89	3.75 ± 4.60	19.57 ± 0.63	18.70 ± 1.70
Cortical cells	4.80 ± 1.68	8.24 ± 3.21	0.00 ± 0.00	1.78 ± 3.86	16.04 ± 1.35	15.32 ± 3.73
Vascular tissue	2.51 ± 1.05	6.30 ± 1.43	1.75 ± 3.84	6.01 ± 4.12	21.18 ± 1.91	13.29 ± 2.82

*No elemental analysis was done due to the absence of the symbiotic zone in winter. Values are means (± SE) of four replications.

Table 6. 6. Elemental composition (relative weight percentage) of different nodule tissues of beach pea: non-significantly distributed elements in winter and summer (Mn, Fe and Zn).

Nodule tissues	Mn		Fe		Zn	
	Winter	Summer	Winter	Summer	Winter	Summer
Meristem	0.00 ± 0.00	0.00 ± 0.00	1.46 ± 2.86	2.38 ± 0.49	1.19 ± 3.17	0.00 ± 0.00
Invasion zone	0.38 ± 1.76	0.40 ± 1.82	0.62 ± 2.28	1.57 ± 2.10	2.81 ± 4.00	0.49 ± 2.03
Symbiotic zone	*	0.54 ± 1.74	*	1.17 ± 2.54	*	0.00 ± 0.00
Senescent zone	1.12 ± 2.49	0.00 ± 0.00	2.97 ± 4.16	3.40 ± 3.44	0.00 ± 0.00	0.00 ± 0.00
Cortical cells	0.00 ± 0.00	0.51 ± 1.72	1.08 ± 2.46	4.21 ± 1.24	1.26 ± 2.65	1.13 ± 2.50
Vascular tissue	0.76 ± 2.10	0.87 ± 2.21	0.65 ± 2.32	2.13 ± 2.57	0.00 ± 0.00	0.66 ± 2.37

*No elemental analysis was done due to the absence of the symbiotic zone in winter. Values are means (± SE) of four replications.

Fig. 6. 1. Photograph of a longitudinal section through a beach pea nodule showing different tissues. Note the meristem (m) at the distal end, invasion zone (i), symbiotic zone (sm), senescent zone (sn), cortical cells (c) and vascular tissue (v).

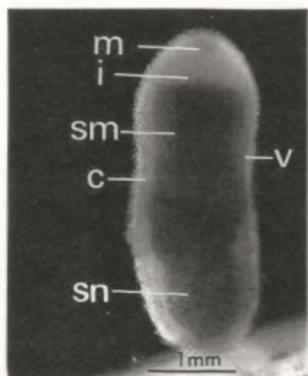


Fig. 6. 2. Seasonal changes in total protein content of beach pea root nodules. Bar values that differed significantly ($P = 0.05$), using Duncan's multiple comparison test, are labeled with different letters. Each bar value is the mean (\pm SE) of three replications.

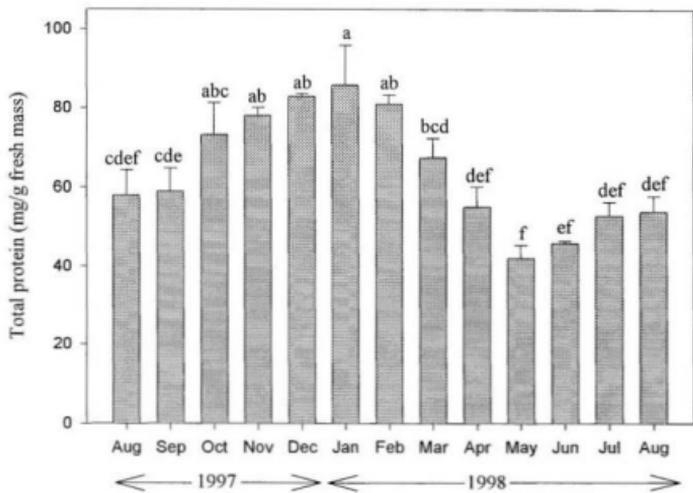


Fig. 6. 3. Seasonal changes in the elemental composition of beach pea nodules: elements which increase in winter. Vertical line on each bar is the standard error.

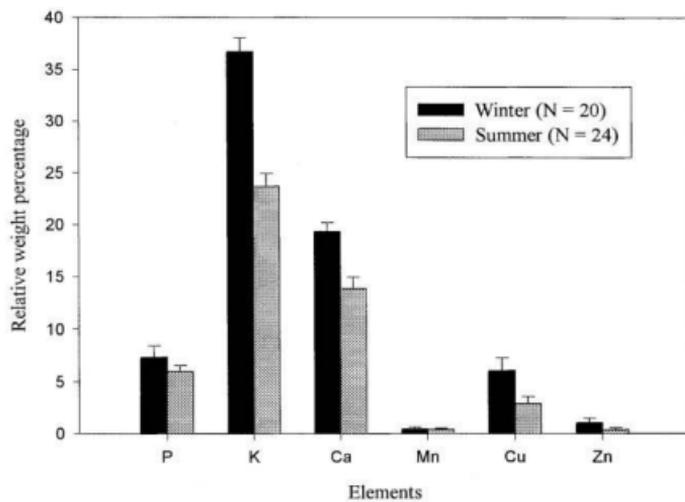
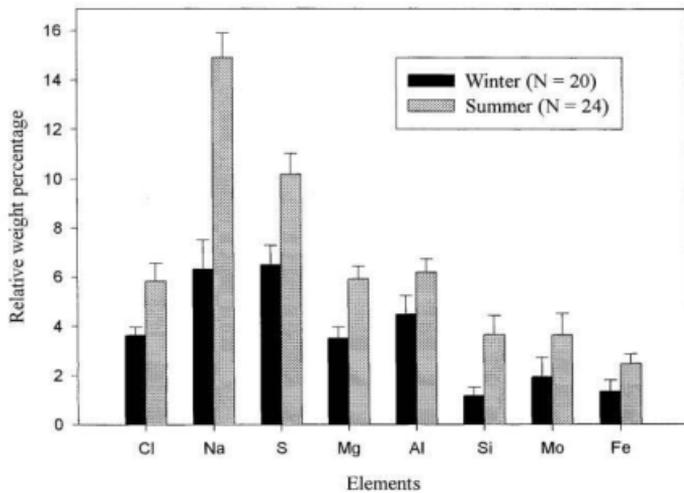


Fig. 6. 4. Seasonal changes in the elemental composition of beach pea nodules: elements which increase in summer. Vertical line on each bar is the standard error.



CHAPTER 7

CHANGES IN DEVELOPING SEEDS OF BEACH PEA AT SIX REPRODUCTIVE GROWTH STAGES

7. 1. Abstract

The developmental patterns of seed, seed coat and hardseededness were studied in native populations of beach pea at six reproductive growth stages. For comparison, grass pea seeds were used in some experiments. The accumulation of fresh and dry weight in pod shell and seed of beach pea and pod shell of grass pea followed almost a sigmoidal pattern. However, grass pea seeds showed a linear pattern of weight accumulation. As maturity advanced, the moisture content of pod shells and seeds decreased due to dehydration. Beach pea seeds started precocious germination at stage 4 (S4). Seeds collected between S1 and S3 failed to germinate due to immaturity, whereas the development of hard seed coats caused ungerminability in seeds gathered at S5 and S6. The imbibition test revealed that hardseededness completely prevented water absorption of S5 and S6 seeds even after 24 days of soaking in water. In grass pea, precocious seed germination was observed at S3, however, the speed of germination, germination percentage, seedling length and dry weight increased as seeds approached maturity. Lipid and protein accumulation in seeds of both crops increased progressively with maturity and showed a positive correlation with seed weight accumulation. In both beach pea and grass pea seeds, S6 was identified as the physiological maturity stage.

7. 2. Introduction

Beach pea seeds possess hard seed coats and do not germinate unless they are scarified (Saw, 1995). There is no information available on the pattern of seed development and maturation in beach pea. In the present work, beach pea and grass pea seeds sampled at six reproductive growth stages, based on the morphology of the pods and seeds, were used. The study proposes: (1) to determine the physical, physiological and biochemical changes in developing and maturing seeds of beach pea and grass pea, and (2) to observe the developmental patterns of the seed coat and development of hardseededness in beach pea.

7. 3. Materials and Methods

7. 3. 1. Plant materials and sampling

7. 3. 1. 1. Beach pea

Developing seeds of beach pea were collected from natural stands on the sandy beach of Salmon Cove, Newfoundland, on 18 August 1999. The classification of the reproductive growth stages was based on the morphology of pods and seeds (Table 7. 1).

7. 3. 1. 2. Grass pea

For comparison, commercially-available grass pea was used in some experiments. The seeds were sown in pots containing sterilized vermiculite on 8 January 1998. In order to maintain a healthy crop, the seedlings were inoculated 13 days after sowing with rhizobial strain USDA 2411 containing 2950×10^5 cells per ml at the rate of 2 ml per

seedling and allowed to grow in a greenhouse at the Atlantic Cool Climate Crop Research Centre, St. John's, Newfoundland. Pods and seeds of each growth stage were collected based on morphological characteristics (Table 7. 2) on 30 April 1998.

7. 3. 2. Estimation of physical changes

7. 3. 2. 1. Fresh and dry weights of pod shells and seeds

Immediately after collection, the pods were rapidly separated into pod shells and seeds. The fresh weights of pod shells and seeds were recorded. After determining the fresh weight, the same pod shells and seeds were allowed to dry at room temperature for about 2 weeks and the dry weight was measured.

7. 3. 2. 2. Moisture content of pod shells and seeds

Both pod shells and seeds were separately sub-sampled for the estimation of moisture content (ISTA, 1993a,b). Randomly-selected samples of pod shells and seeds were dried, after recording the fresh weight, at 105 °C in an oven for 16 ± 1 hours. After cooling for 30 minutes at room temperature, the dry weight of the samples was recorded. The moisture content was calculated and expressed as a percentage by using the following formula:

$$\text{Moisture content} = \frac{M_2 - M_3}{M_2 - M_1} \times 100$$

where,

M_1 = Weight of the glass vial alone

M_2 = Weight of the glass vial + sample before drying

M_3 = Weight of the glass vial + sample after drying

7. 3. 3. Estimation of physiological changes

7. 3. 3. 1. Speed of germination

One hundred seeds in each replicate were allowed to germinate in a petri dish with moistened filter paper at room temperature. The sprouted seeds were counted daily from sowing until 24 days. From the number of seeds germinated on each day, the speed of germination (SOG) was calculated using the following modified formula (Maguire, 1962):

$$\text{SOG} = \frac{X_1}{Y_1} + \frac{X_2 - X_1}{Y_2} + \dots + \frac{X_n - (X_n - 1)}{Y_n}$$

where,

X_n = Number of seeds germinated at n^{th} count

Y_n = Number of days from sowing to n^{th} count

7. 3. 3. 2. Germination percentage

The germination test was conducted according to ISTA with minor modifications (ISTA, 1993a,b). One hundred seeds in each replicate were sown in plastic buckets containing sterilized vermiculite and allowed to grow in controlled environment chambers with a day/night period of 16 hours/8 hours at 16 °C/8 °C and irrigated with

regular tap water. At the end of the 24th day, the number of normal seedlings was counted and the percentage of germination was calculated.

7. 3. 3. 3. Seedling length and dry weight

Ten normal seedlings per replication were randomly taken from each sample at the end of the germination test. The distance between the tip of the plumule and primary root was measured and the mean value was expressed as seedling length in centimeters. For dry weight estimation, the seedlings used for the length measurements were dried in an oven maintained at 105 °C for 72 hours. Then the seedlings were cooled at room temperature for 30 minutes. The dry weight was measured.

7. 3. 4. Estimation of biochemical changes

The dried seeds were ground to a powder and used for the extraction and estimation of total lipid content as mentioned in chapter 3 (see section 3. 3. 5 on page number 44). The total protein content was determined as described in chapter 6 (see section 6. 3. 2 on page number 91).

7. 3. 5. Microscopical observations of seed coat development in beach pea

7. 3. 5. 1. Light microscopy

Developing and mature seeds were cut into small pieces using a clean razor blade. The samples were fixed in formalin-acetic acid-alcohol (FAA) for 2 days, dehydrated in a graded ethanol and tertiary butyl alcohol (TBA) series and embedded in paraffin wax

(Jensen, 1962). Serial sections, 5 – 7 μm in thickness, were cut on an AO 820-Spencer microtome (American Optical Corporation, Buffalo, New York, USA) and mounted on slides using albumin fixative (Fisher Scientific Company, New Jersey, USA). They were stained with Safranin-Fast Green (Jensen, 1962) and photomicrographs were taken with a Zeiss AXIOMAT photomicroscope.

7. 3. 5. 2. Scanning electron microscopy

Scanning electron microscopy was performed on hand-cut sections of developing seeds of beach pea after air-drying. The samples were mounted on aluminum stubs with two-sided carbon adhesive tape (J.B. EM Services Inc., Dorval, Quebec, Canada) and sputter coated with gold on an Edwards S150 sputter coater (Edwards, West Sussex, England). The coated samples were viewed with a Hitachi S570 scanning electron microscope at an accelerating voltage of 20 kV.

7. 3. 6. Determination of hard seed coat development in beach pea

7. 3. 6. 1. Water imbibition test

Five seeds in dry state were taken at random from every growth stage, except S1, and the initial weight of individual seeds was recorded. Due to their very small size, S1 seeds were not suitable for this experiment. Each seed was placed separately in 10 ml of distilled water at room temperature and was kept in the dark. The seeds were removed from water, dried with tissue paper, weighed and returned to the same water. The rate of water uptake was measured at 1, 2, 4, 6 and 12 hour(s), and also at 1, 2, 3, 4, 7, 10, 15, 20

and 24 day(s). The water imbibition (Legesse and Powell, 1996) was calculated by using the following formula:

$$\text{Water imbibition (\%)} = \frac{\text{weight of imbibed seed at a particular time} - \text{initial weight of seed}}{\text{initial weight of seed}} \times 100$$

7. 3. 6. 2. Tetrazolium chloride test

Ungerminated seeds, remaining in all growth stages, at the end of the 24th day of the germination experiment were tested for viability using the tetrazolium chloride test. After removal of the seed coat, the cotyledon and embryo were immersed in a 0.1% solution of 2, 3, 5 triphenyl tetrazolium chloride at room temperature in the dark for 2 hours (ISTA 1993a, b). The seeds stained red colour were considered viable and were photographed.

7. 3. 7. Statistical analysis

For all sets of data, one-way analysis of variance was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test. For the purpose of statistical analysis, data in percentage were transformed to arcsine values (Snedecor and Cochran, 1980; Zar, 1996).

7. 4. Results

7. 4. 1. Physical and physiological changes

Figures 7. 1 and 7. 2 show six reproductive growth stages of pods and seeds of beach pea and grass pea, respectively. Fresh and dry weight of pod shell in both beach pea and grass pea increased from S1 to S5 with a rapid increase between S1 and S2 and decreased between S5 and S6 (Table 7. 3). The fresh and dry weight of seeds also increased rapidly with the advancement of maturity and reached a maximum at S5 in beach pea and S6 in grass pea (Table 7. 4). The rate of accumulation of fresh and dry weight of seeds in beach pea was higher between S2-S3 and S3-S4 respectively, whereas grass pea seeds showed a greater rate of accumulation of both fresh and dry weights between S1 and S2. In both species, the pattern of growth and development of pod shells and seeds were almost the same and followed a sigmoidal pattern except for grass pea seeds, which showed a linear pattern.

After a slight increase between S1 and S2, beach pea pod shells and seeds showed a gradual reduction in moisture content until S5, and rapid water losses of about 57% in pod shells and 46% in seeds were recorded between S5 and S6. In grass pea, the pod shell moisture content increased between S1 and S3 and decreased between S4 and S6, whereas seed moisture content increased between S1 and S2 and dropped between S3 and S6. As in beach pea, grass pea also lost a greater amount of water between S5 and S6 which accounted for about 41% in pod shells and 48% in seeds.

Beach pea seeds were shown to be ready for precocious germination at S4 (Table 7. 5). There was no germination of seeds collected from other reproductive growth stages.

Grass pea seeds started precocious germination at S3 with 56.67% and it increased as maturity advanced and reached a maximum of 96.67% at S5. A reduction in the percentage of germination was observed between S5 and S6 due to dehydration. However, germination speed, seedling length and seedling dry weight increased progressively with maturity and reached a maximum at S6 (Table 7. 5).

7. 4. 2. Biochemical changes

Seeds collected at S1 in both species were very small and not sufficient for biochemical analyses. As maturity advanced, lipids accumulated gradually and reached a maximum at S6 in both beach pea and grass pea seeds (Table 7. 6). The highest rates of lipid deposition of about 36% in beach pea seed and 43% in grass pea seed were recorded between S2 and S3. Protein accumulation in seeds of both crops increased linearly until the final stage (Table 7. 6). The rate of seed protein accumulation was highest between S3 and S4 (29%) in beach pea and S2 and S3 (41%) in grass pea.

7. 4. 3. Microscopical observations of beach pea seed coat development

Observations of S2 seeds of beach pea with the light microscope revealed the dividing epidermal layer without any apparent differentiation (Fig. 7. 3). This layer later gave rise to palisade cells. The different layers of the mature seed, such as palisade cells, hourglass cells and parenchyma cells, were not evident at this stage. Developing and differentiating round cells with large vacuoles were seen in the subepidermal zone. At S3, seeds had developed most of the seed coat layers found in the mature seeds (Figs. 7. 4A

and 7. 4B). However, cells in these layers were immature and still undergoing differentiation. Cellular contents at this stage were very dense and the seed internal mass increased as indicated by the increased cell size. As the cotyledons elongate at S5, the endosperm was compressed and reduced to form a single layer, which remained as remnants between parenchyma cells and cotyledons (Fig. 7. 5A). The hourglass cells showed changes in their shape and thickness (Fig. 7. 5B).

Mature seeds collected at S6 showed a papillose surface pattern (Fig. 7. 6A) on the seed coat with many ribs or waxy incrustations as noted by Chernoff et al. (1992) in other legume seeds. Cross section through S6 mature seed showed thick palisade cells, compressed hourglass cells, well-developed parenchyma cells and cotyledons (Fig. 7. 6B). Figure 7. 6C shows a surface view of the mature seed with well-developed hilar groove, median fissure and micropyle. Transverse section through hilar region in a mature seed at S6 showed completely developed hilar tissues (Fig. 7. 6D). Tracheids appeared as a tear-drop shaped zone containing radial files of cells. It also revealed a widely differentiated epidermis as a counter palisade layer and palisade layer. The ends of the tracheids were surrounded by sclerenchyma and then parenchyma.

7. 4. 4. Hard seed coat development in beach pea

The rate of water imbibition in the developing seeds declined as maturity advanced (Fig. 7. 7). Seeds collected at S5 and S6 did not absorb water at all, even after 24 days of imbibition, due to the impermeable seed coats. The pattern of water imbibition was not uniform throughout the test period. It was rapid during the first 12 hours.

Tetrazolium chloride test, by forming red staining in living cells, proved that the seeds were viable even after 24 days of soaking (Fig. 7. 8). This indicates that the ungerminability was due to hard seed coats and the lack of water imbibition.

7. 5. Discussion

In most studies, days after flowering have been the criterion for sampling in developing seeds of many plants. Fehr et al. (1971) and Dornbos and McDonald (1986) proposed a method for describing soybean seed development using reproductive growth stages that were based on plant morphological descriptions. Seed development rate differs according to genotype and environmental conditions, leading to variable days after flowering values for the same seed development stage. Morphologically-described growth stages, in contrast, allow a precise, rapid and convenient sampling method for seed development and composition studies. Bewley and Black (1978) have mentioned that the rate of seed development in sampling methods based on days after flowering could not be compared because, although the pattern of seed development was consistent, the timing was not. In this investigation, therefore, morphologically-described growth stages were used as the beach pea samples were collected from naturally-growing stands.

The increased accumulation of fresh and dry weight of pod shells and seeds in both beach pea and grass pea at early stages of development reflects the rapid cell division and elongation and deposition of storage reserves. The simple sigmoidal curves demonstrated an almost perfect synchronization of accumulation of fresh and dry weight in pod shells and seeds. This interrelation between fruit and seed development was

similar to those reported in other crops (Dure, 1975; Gurusamy and Thiagarajan, 1998). The linear pattern of weight accumulation in grass pea seeds is most likely due to delayed termination of reserve accumulation. It may also be due to lack of fluctuation in growth conditions as these plants were grown in controlled greenhouse conditions.

Seed maturation is generally terminated by some degree of drying, which results in a gradual reduction in metabolism as water is lost from the seed tissues and the embryo passes into a metabolically inactive or quiescent state (Kermode, 1990). Physiological maturity denotes the stage of development when the seed reaches its maximum dry weight and marks the end of the seed-filling period (Shaw and Loomis, 1950). In the present study, maximum dry weight of beach pea seeds was obtained at S5 when the moisture content of the seed reached 73%. However, there was no significant change in seed dry weight between S5 and S6, but the moisture content dropped to 27%. This indicates that S6 is the physiological maturity stage. Grass pea seeds reached physiological maturity at S6 with maximum dry weight and 32% moisture content.

Higher moisture content in pod shells and seeds at initial stages might be due to less dehydration. The loss of water during maturation is an inherent phase of development (McIlrath et al., 1963). As pointed out by Sreeramulu et al. (1992) as well as Gurusamy and Thiagarajan (1998), the decrease in moisture content of pod shells and seeds observed in the present work as maturity advanced is probably due to the utilization of water in various metabolic activities and the removal of water by desiccation caused by surrounding environment. Previous research shows that the reduction of pod and seed weight at the physiological maturity stage might be due to oxidation and volatilization

(Harrington, 1972), desiccation (Rao and Rao, 1975; Kermodé, 1990) and loss of nutrients (Rauf, 1978; Sreeramulu et al., 1992).

Seed maturation is not necessary for germinability. The seed, or the embryo therein, is capable of germinating during development (Bewley and Black, 1994). This phenomenon is called precocious germination. In the present investigation, beach pea seeds started germination precociously at S4, but seeds from other stages did not germinate. Seeds collected from S1 to S3 failed to germinate probably due to immaturity. This was further proved by increased water imbibition of these seeds as a consequence of poorly-developed seed coats. According to Corner (1951), tracheids may function as a water reservoir during the development of seed. The hilum has been reported as the site of water entry in the seeds of common vetch (Aswathaiyah, 1988). The reduced rate of water imbibition at S4 indicates the development of hardseededness. Later at S5 and S6, the seeds became harder and impermeable, which was evident from an absence of water absorption and a failure to germinate.

Several researchers attributed hardseededness to a lack of grooves in palisade cells, unbroken and more prominent light lines (Serrato-Valenti et al., 1989), environmental influence (Tomer and Kumari, 1991), a thick and continuous cuticle (Russi et al., 1992), and a higher deposition of cutin (Seth and Vijayaraghavan, 1990), fiber, lignin, silica (Rodriguez and Mendoza, 1990), tannins (Stanley, 1992), phenolics, pectins (Holubowicz et al., 1988), phytate, divalent cations (Ca, Mg) and proteins (Shehata, 1992) in seed coats. Seed coat impermeability to water has been attributed to one or more of the following structures: strophiole (Gopinathan and Babu, 1985), chalaza

(Spurny, 1972), micropyle (Bhattacharya and Saha, 1990), adherence of the seed coat to cotyledons (Legesse and Powell, 1996) and remnants of the inner integuments (Roti-Michelozzi et al., 1987). In beach pea seeds, Chavan (1998) has detected the presence of phenolics and tannins; the accumulation of which may be partly responsible for the impermeable hardseededness. Further biochemical studies of isolated seed coats are necessary to characterize the seed coat components.

Grass pea seeds showed precocious germination at S3, but both germination percentage and its speed increased with increase in maturity. This might be due to the fact that during early development the embryos initially lack sufficient nutrients to support their continued development to a germinable stage, and also lack the nutrients and stored reserves to support germination and post-germination growth (Kermode, 1990; Bewley and Black, 1994). Viable seedlings in terms of seedling length and dry weight increased with maturity. Similar results were previously reported in legumes (Ellis et al., 1987) and in cauliflower (Gurusamy and Thiagarajan, 1998).

Lipid and protein content of seeds increased steadily as maturity advanced and showed positive correlation with seed weight in beach pea and grass pea and also with seedling vigour parameters in grass pea. The rapid accumulation of lipids and proteins at early stages indicated the deposition of storage reserves due to the availability of plenty of appropriate enzymes or precursors for lipid and protein syntheses (Bewley and Black, 1994). The reduction in the rate of accumulation of lipids and proteins at later stages might be due to the gradual decline in metabolic activities as desiccation sets in (Kermode, 1990). These results are in agreement with the findings reported in soybean

(Dornbos and McDonald, 1986). Physiological maturity is the most suitable stage at which seeds should be harvested (Ellis et al., 1987), because seeds achieve maximum viability and vigour at this stage since nutrients are no longer entering the seed from the plant, and thereafter seeds begin to age (Harrington, 1972). In the present work, based on the dry weight of seed and accumulation of lipids and proteins, S6 was identified as the physiological maturity stage in both beach pea and grass pea.

Table 7. 1. Stages of seed development in beach pea based on the morphological characteristics of pods and seeds.

Growth stages	Pod length (cm)	Pod width (cm)	Pod colour	Seed colour
S1	1.5 – 3.9	0.2 – 0.5	Purple with green border	Pale green
S2	4.0 – 5.0	0.6 – 0.9	Purple with green border	Green
S3	5.1 – 6.0	1.0 – 1.2	Green with light purple patches	Green
S4	5.1 – 6.0	1.0 – 1.2	Green	Green
S5	5.1 – 6.0	1.0 – 1.2	Greenish pale yellow with purple patches	Green
S6	5.1 – 6.0	1.0 – 1.2	Dark brown	Dark brown or black

Table 7. 2. Stages of seed development in grass pea based on the morphological characteristics of pods and seeds.

Growth stages	Pod length (cm)	Pod width (cm)	Pod colour	Seed colour
S1	0.9 – 2.2	0.3 – 0.9	Green	Yellow green
S2	2.3 – 2.8	1.0 – 1.3	Green	Green
S3	2.9 – 3.4	1.3 – 1.4	Green	Green
S4	3.5 – 4.3	1.5 – 1.6	Green	Green
S5	3.5 – 4.3	1.5 – 1.6	Green with pink spots	Green
S6	3.5 – 4.3	1.5 – 1.6	Pale yellow or brown pods with dark brown spots on one side	Dull white brown with dark brown or black spots

Table 7. 3. Changes in the pod shell weight and moisture content of beach pea and grass pea at six reproductive growth stages.

Growth stages	Fresh weight (mg/pod shell)		Dry weight (mg/pod shell)		Moisture content (%)*	
	Beach pea (N = 4)	Grass pea (N = 3)	Beach pea (N = 4)	Grass pea (N = 3)	Beach pea (N = 4)	Grass pea (N = 3)
S1	62.75 ± 16.77 ^d	68.33 ± 22.40 ^d	12.25 ± 3.43 ^d	19.60 ± 5.28 ^e	84.05 ± 0.71 ^a	68.75 ± 4.78 ^a
S2	218.00 ± 41.59 ^c	171.43 ± 5.47 ^d	37.00 ± 6.49 ^d	54.47 ± 4.97 ^d	84.24 ± 0.75 ^a	71.26 ± 0.37 ^a
S3	383.00 ± 61.10 ^b	330.33 ± 58.78 ^c	72.75 ± 15.21 ^c	116.50 ± 11.03 ^c	81.36 ± 1.11 ^b	75.37 ± 0.85 ^a
S4	439.00 ± 33.77 ^b	460.00 ± 37.65 ^b	104.25 ± 6.59 ^{bc}	164.60 ± 13.87 ^b	79.19 ± 0.34 ^b	72.87 ± 0.41 ^a
S5	626.25 ± 40.11 ^a	597.10 ± 27.13 ^a	159.25 ± 22.42 ^a	190.93 ± 5.30 ^a	79.27 ± 0.23 ^b	71.66 ± 0.75 ^a
S6	185.75 ± 61.45 ^{cd}	162.27 ± 25.24 ^d	124.50 ± 4.94 ^{ab}	132.87 ± 6.40 ^c	22.09 ± 0.42 ^c	30.83 ± 9.47 ^b

* Arcsine transformed values were used for statistical analyses.

Mean ± SE followed by different letters in the same column are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Table 7. 4. Changes in the seed weight and moisture content of beach pea and grass pea at six reproductive growth stages.

Growth stages	Fresh weight (mg/seed)		Dry weight (mg/seed)		Moisture content (%)*	
	Beach pea (N = 4)	Grass pea (N = 3)	Beach pea (N = 4)	Grass pea (N = 3)	Beach pea (N = 4)	Grass pea (N = 3)
S1	0.53 ± 0.22 ^c	1.07 ± 0.69 ^d	0.20 ± 0.05 ^c	0.33 ± 0.16 ^c	82.76 ± 2.14 ^a	80.02 ± 0.87 ^a
S2	1.74 ± 0.28 ^c	18.04 ± 8.02 nd	0.40 ± 0.03 ^c	3.72 ± 1.86 ^c	86.80 ± 0.14 ^a	84.86 ± 1.23 ^a
S3	9.50 ± 4.62 ^c	49.51 ± 8.24 ^c	1.69 ± 0.82 ^c	10.81 ± 2.18 ^{bc}	86.71 ± 0.77 ^a	81.73 ± 0.47 ^a
S4	38.07 ± 8.02 ^b	98.00 ± 13.28 ^b	7.84 ± 2.23 ^b	23.43 ± 2.63 ^{bc}	84.75 ± 1.03 ^a	81.07 ± 0.22 ^a
S5	88.01 ± 4.58 ^a	123.23 ± 25.37 ^{ab}	32.17 ± 1.34 ^a	34.23 ± 11.72 ^b	73.12 ± 2.72 ^b	80.14 ± 0.49 ^a
S6	49.73 ± 11.50 ^b	155.67 ± 19.12 ^a	31.43 ± 1.47 ^a	120.90 ± 13.22 ^a	27.39 ± 4.07 ^c	32.06 ± 7.89 ^b

* Arcsine transformed values were used for statistical analyses.

Mean ± SE followed by different letters in the same column are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Table 7. 5. Changes in the physiological vigour parameters of beach pea and grass pea seeds at six reproductive growth stages (N = 3).

Growth stages	Speed of germination		Germination (%) ^a		Seedling length (cm)		Seedling dry weight (mg)	
	Beach pea	Grass pea	Beach pea	Grass pea	Beach pea	Grass pea	Beach pea	Grass pea
S1	0.00 ± 0.00 ^b	0.00 ± 0.00 ^e	0.00 ± 0.00 ^b	0.00 ± 0.00 ^e	0.00 ± 0.00 ^b	0.00 ± 0.00 ^d	0.00 ± 0.00 ^b	0.00 ± 0.00 ^f
S2	0.00 ± 0.00 ^b	0.00 ± 0.00 ^e	0.00 ± 0.00 ^b	0.00 ± 0.00 ^e	0.00 ± 0.00 ^b	0.00 ± 0.00 ^d	0.00 ± 0.00 ^b	0.00 ± 0.00 ^f
S3	0.00 ± 0.00 ^b	0.28 ± 0.02 ^d	0.00 ± 0.00 ^b	56.67 ± 1.92 ^d	0.00 ± 0.00 ^b	5.00 ± 1.24 ^c	0.00 ± 0.00 ^b	12.00 ± 2.08 ^d
S4	0.18 ± 0.06 ^a	0.37 ± 0.02 ^c	32.50 ± 1.92 ^a	73.33 ± 2.21 ^c	21.63 ± 0.98 ^a	22.03 ± 2.60 ^b	8.75 ± 3.04 ^a	29.00 ± 4.51 ^c
S5	0.00 ± 0.00 ^b	0.64 ± 0.02 ^b	0.00 ± 0.00 ^b	96.67 ± 6.14 ^a	0.00 ± 0.00 ^b	28.77 ± 1.19 ^b	0.00 ± 0.00 ^b	42.00 ± 2.65 ^b
S6	0.00 ± 0.00 ^b	0.92 ± 0.02 ^a	0.00 ± 0.00 ^b	86.67 ± 2.71 ^b	0.00 ± 0.00 ^b	46.37 ± 6.13 ^a	0.00 ± 0.00 ^b	78.00 ± 3.22 ^a

^a Arcsine transformed values were used for statistical analyses.

Mean ± SE followed by different letters in the same column are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Table 7. 6. Changes in total lipids and proteins of beach pea and grass pea seeds at six reproductive growth stages (N = 3).

Growth stages	Total lipids (mg/g dry weight)		Total proteins (mg/g dry weight)	
	Beach pea	Grass pea	Beach pea	Grass pea
S1	*	*	*	*
S2	14.93 ± 0.75 ^e	8.73 ± 0.38 ^e	111.23 ± 6.08 ^d	81.37 ± 4.87 ^d
S3	20.37 ± 0.39 ^d	12.47 ± 0.41 ^d	135.80 ± 2.95 ^d	114.40 ± 5.13 ^c
S4	25.83 ± 0.90 ^c	16.40 ± 0.75 ^c	175.57 ± 6.68 ^c	136.53 ± 2.51 ^b
S5	31.94 ± 0.86 ^b	19.13 ± 0.47 ^b	201.10 ± 5.10 ^b	154.57 ± 3.64 ^a
S6	34.82 ± 0.43 ^a	20.93 ± 0.27 ^a	230.49 ± 13.77 ^a	167.67 ± 5.50 ^a

*Not determined.

Mean ± SE followed by different letters in the same column are significantly different using Duncan's multiple comparison test at $P = 0.05$.

Fig. 7. 1. Six reproductive growth stages of pods (A) and seeds (B) in beach pea. For explanation of stages, see Table 7. 1.

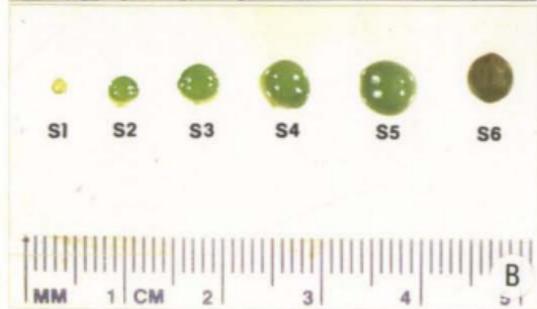


Fig. 7. 2. Six reproductive growth stages of pods (A) and seeds (B) in grass pea. For explanation of stages, see Table 7. 2.



Fig. 7. 3. Cross section of beach pea seed at stage 2. Note the undifferentiated epidermal layer (el) and round cells with large vacuoles (arrows).

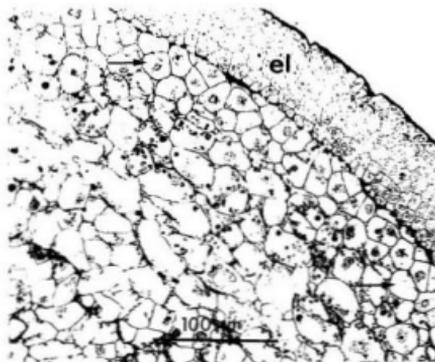


Fig. 7. 4. Beach pea seed at stage 3. A. Scanning electronmicrograph showing the immature seed coat of beach pea at S3. Note young palisade cells (pc), long and thin hourglass cells (hgc), and developing parenchyma cells (p). B. Photomicrograph showing well-differentiated cell layers of beach pea seed at S3. (c = cuticle, pc = palisade cells, hgc = hourglass cells, p = parenchyma).

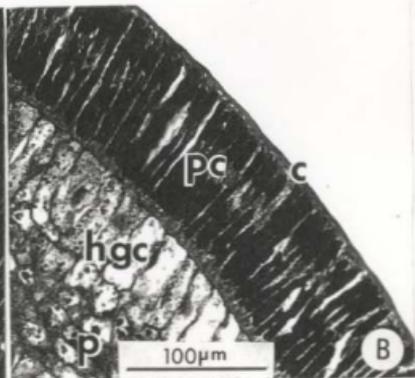
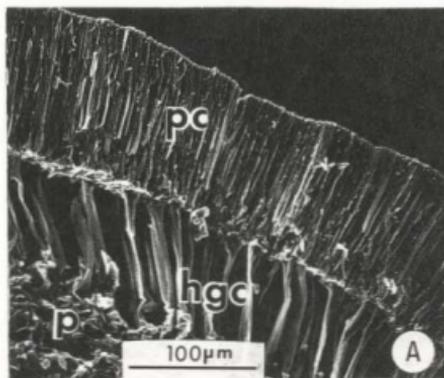


Fig. 7. 5. Photomicrograph showing cross section of beach pea seed at stage 5. A. As cotyledons develop, the endosperm is gradually utilized and left as remnants (arrow) between parenchyma cells (p) and cotyledons (co). (c = cuticle, pc = palisade cells, hgc = hourglass cells, em = embryo). B. Higher magnification of beach pea seed coat shown in Fig. 7. 5A illustrating changes in the shape and thickness (arrow) of hourglass cells (hgc).

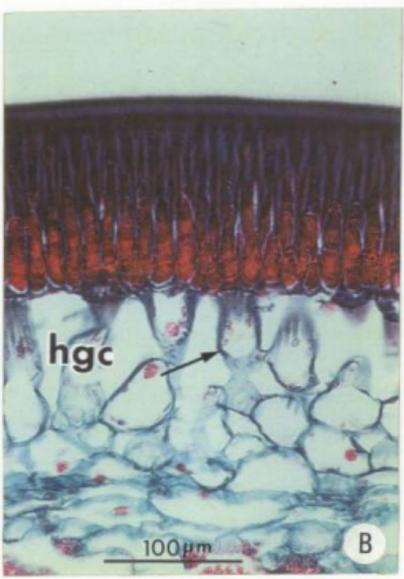
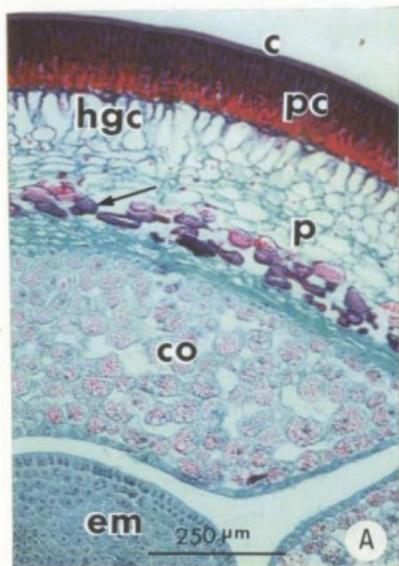


Fig. 7. 6. Structural features of mature beach pea seed at stage 6. A. Scanning electronmicrograph showing papillose surface pattern of seed coat in mature beach pea seed. B. Scanning electronmicrograph of mature seed of beach pea showing well-developed palisade cells (pc), thick and compressed hourglass cells (hgc), mature parenchyma (p) and cotyledons (co). C. Scanning electronmicrograph showing surface view of hilum in mature beach pea seed. (hg = hilar groove, mf = median fissure, m = micropyle). D. Photomicrograph of transverse section of mature beach pea seed passing through hilum. Hilum tissues include hilar groove (hg), counter palisade layer (CL), palisade layer (PL), tracheids (t), sclerenchyma (SC) and parenchyma (P).

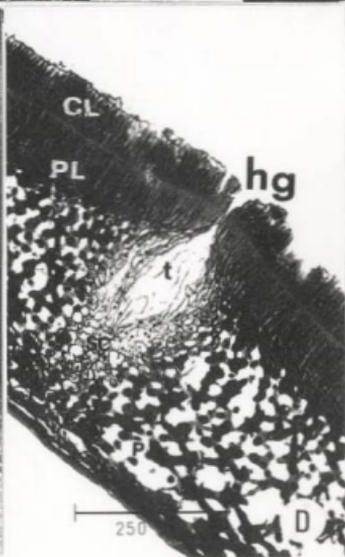
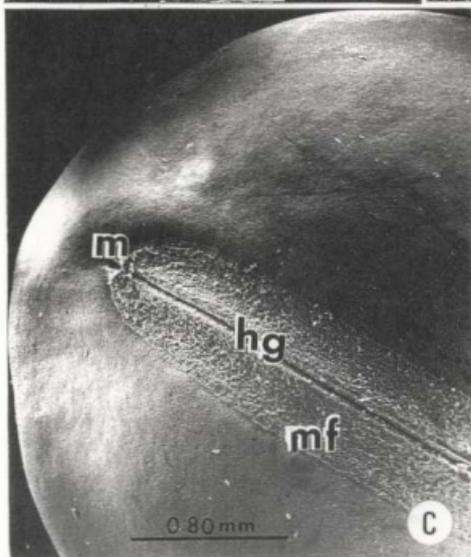
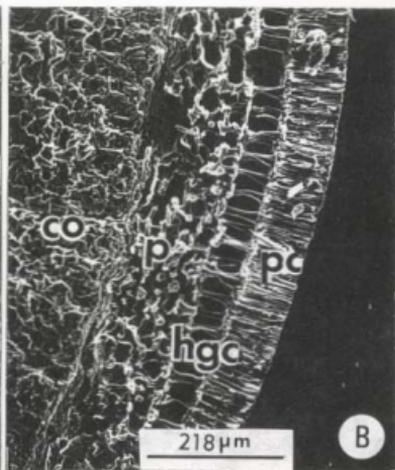
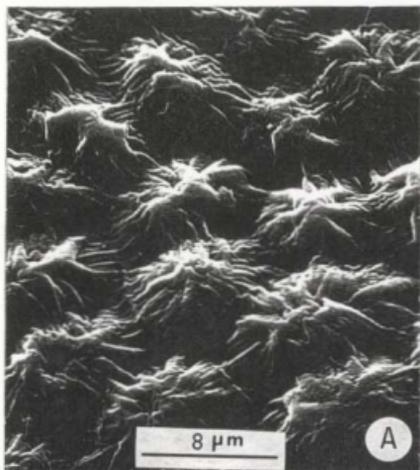


Fig. 7. 7. The pattern of water imbibition by beach pea seeds at different stages of development. S1 seeds were not used due to their small size. Seeds collected at S5 and S6 did not absorb water throughout the experiment. Each value is the mean (\pm SE) for five replications.

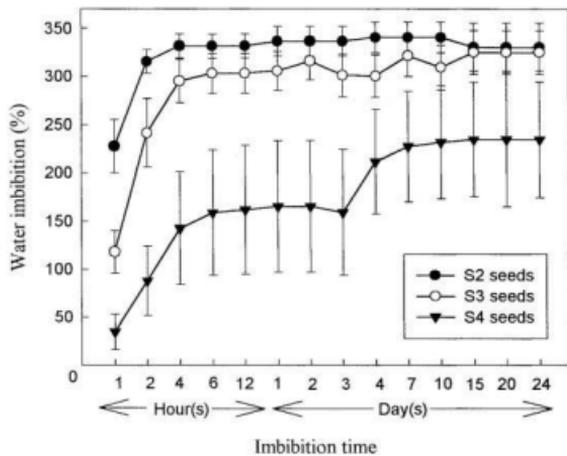
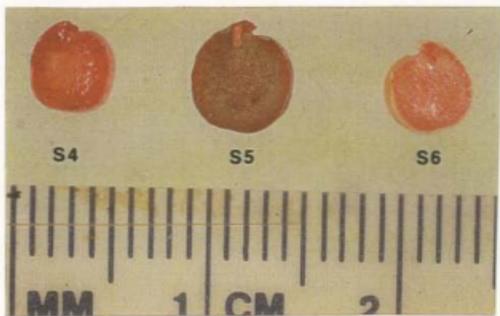


Fig. 7. 8. Photograph showing viability of ungerminated beach pea seeds of S4, S5 and S6 at 24 days after germination experiment. Seeds were treated with 0.1% tetrazolium chloride for 2 hours after removing seed coat. A red colour indicates living cells.



CHAPTER 8

EFFECT OF SEED ABORTION ON THE PATTERN OF SEED FORMATION IN BEACH PEA

8. 1. Abstract

The pattern of seed abortion in the developing pods of beach pea was studied at six reproductive growth stages and compared to seed abortion in grass pea. In both crops, the frequency distribution of developing seeds did not match that of ovules per ovary, and cryptic seed abortion started early in pod ontogeny and reached a maximum at stage 2 (S2). Only 68 % and 82 % of ovules produced mature seeds in beach pea and grass pea, respectively. The pattern of seed abortion and development within developing pods was random in beach pea and nonrandom in grass pea. Ovules located in position 1 and 2 at the basal region of pods showed higher seed abortion in beach pea. In grass pea, the probability of seed abortion increased towards the basal end of the pod.

8. 2. Introduction

The yield is mainly determined by number of pods and seeds in grain legumes. Pod and seed losses occur through abortion of flowers, pods and seeds, which generally coincides with a cessation of embryo development occurring at different stages either for all or only some of the seeds in a pod (Linck, 1961; Abernathy et al., 1977). Most of the studies on seed abortion were carried out in leguminous plants (Ganeshaiah and Uma Shaanker, 1988; Uma Shaanker and Ganeshaiah, 1988; Rocha and Stephenson, 1991;

O'Donnell and Bawa, 1993; Mohan Raju et al., 1995) and a few in cruciferous plants (Mazer et al., 1986; Stanton, 1987; Nakamura and Stanton, 1987; Gurusamy, 1999b) and graminaceous plants (Zhao and Chen, 1990). According to Hossaert and Valero (1988), linear fruits of leguminous plants offer convenient opportunities for seed abortion studies due to two opposing gradients, namely, a gradient in distance from maternal resources, from the basal to stylar end, and a gradient in distance from pollen deposited on the stigma, from the stylar to basal end (Labeyrie and Hossaert, 1985).

Most of the seed abortion studies were conducted on mature or nearly mature fruits and over the course of seed development at different days after anthesis (Stanton, 1987; Gurusamy, 1999b). Many authors, however, have suggested seed development studies based on morphologically-described growth stages as the best method (Fehr et al., 1971; Dornbos and McDonald, 1986).

In the present work, beach pea and grass pea seeds sampled at six reproductive growth stages, based on the morphology of pods and seeds, were used. The study proposes: (1) to determine the pattern of seed formation in relation to seed abortion and (2) to identify the effects of ovule position on both seed abortion and maturation.

8. 3. Materials and Methods

8. 3. 1. Plant materials and sampling

Developing seeds of beach pea and grass pea were collected (refer to Tables 7. 1 and 7. 2) as described in chapter 7 (see section 7. 3. 1 on page number 112).

8. 3. 2. Measurements of pod and seed development

Pods collected at the six growth stages were dissected and the numbers of ovules, aborting seeds and developing seeds were recorded. The dry weights of pod shells and seeds were recorded 2 weeks after drying at room temperature. The seed-to-ovule ratio at each growth stage was determined by dividing the number of developing seeds by the total number of ovules per pod.

8. 3. 3. Frequency distribution of ovules and seeds per pod

To estimate the frequency distribution, pods collected at different growth stages were used to count total numbers of ovules per ovary and developing seeds per pod.

8. 3. 4. Estimation of probability of seed abortion and development at different ovule positions

The ovule positions were assigned by numbering from the basal end (first position) to stylar end (last position). The number of ovules in each pod was standardized to compare the rate of seed abortion and probability of seed development among pods having different ovule numbers by using the transformation formula described by Hossaert and Valero (1988):

$$P = 10 F / N \quad \text{for beach pea}$$

$$P = 3 F / N \quad \text{for grass pea}$$

where,

P = The new ovule position

F = The former ovule position

N = The total number of ovules in a pod

The percentage of seed abortion for each position in the pod at different growth stages was calculated by dividing the number of aborted seeds by total number of ovules in that position. The probability of seed development for each position in the pod at different growth stages was also calculated by dividing the number of developing seeds by the total number of ovules in that position.

8. 3. 5. Statistical analysis

For all data sets, one-way analysis of variance was performed using the SPSS computer package (SPSS Inc., 1990). Means were compared by Duncan's multiple comparison test at $P = 0.05$.

8. 4. Results

As pods matured, the dry weights of the pod shells and seeds in beach pea (Table 8. 1) and the dry weight of the pod shell in grass pea (Table 8. 2) increased until S5 and decreased between S5 and S6. The rate of dry weight accumulation was higher in beach pea pod shells between S1 and S2 (about 201%), whereas it was greater in seeds between S3 and S4 (about 361%). A reduction of about 21%, 2% and 30% was observed in dry weights of pod shells and seeds in beach pea and pod shells in grass pea, respectively,

between S5 and S6. However, the accumulation of seed dry weight in grass pea showed a linear pattern with a maximum gain of about 994% between S1 and S2. In both crops, aborted seeds were small and yellow in colour. Figure 8. 1 shows aborted seeds in beach pea pod at S4. Developing seeds were full size, green before maturation and dark brown or black in beach pea and brown with black spots in grass pea at maturation.

In beach pea, seed abortion showed non-significance between growth stages (Table 8. 1). However, a maximum of about 34% of seed abortion was observed at S2 and S3. Mature pods collected at S6 showed 31.5% seed abortion. Both developing seeds per pod and seed-to-ovule ratio were also not significantly different between growth stages. Number of ovules plus seeds per pod increased until S2 and showed a slight decline in later growth stages. In grass pea, 24.9% of ovules in a pod were aborted at S1 (Table 8. 2). The seed abortion increased significantly between S1 and S2 and then declined as pods matured. A maximum seed abortion of 48.5% was observed in a pod at S2. The last three growth stages showed significantly higher numbers of developing seeds per pod and seed-to-ovule ratio compared to the first three growth stages, whereas the number of ovules plus seeds per pod increased steadily as pods matured.

The overall number of ovules in all pods, from the six growth stages, ranged from 7 to 13 with a mean of 10 in beach pea and from 2 to 4 with a mean of 3 in grass pea. Figures 8. 2 – 8. 5 show the shift in the frequency distribution of ovules per ovary and seeds per pod at different growth stages in beach pea and grass pea. In both crops, the distribution pattern of developing seeds did not correspond to that of ovules per ovary as a consequence of seed abortion. The number of seeds per pod at different growth stages

ranges from 2 to 10 in beach pea and 1 to 4 in grass pea. After standardizing the numbers of ovules in a pod at 10 in beach pea and 3 in grass pea, the distribution of aborted seeds in pods of different growth stages was random in beach pea (Fig. 8. 6) and nonrandom in grass pea (Fig. 8. 7). Overall, the probability of seed abortion in beach pea was high in ovule position 1 followed by position 2. In grass pea, the probability of seed abortion increased towards the basal end of the pod at all growth stages.

Beach pea and grass pea pods also showed random and nonrandom patterns of seed development at different growth stages, respectively (Figs. 8. 8 and 8. 9). In beach pea, the probability of seed development was less in ovule positions 1 and 2 compared to other positions. Irrespective of growth stages, position 5 recorded more chances for seed development (87.5%) followed by position 9 (85.8%). In grass pea, the probability of seed development showed an increased gradient towards the stylar end of the pod. Position 3 showed more chances for seed development registering 75.7 – 93.7% at different growth stages.

8. 5. Discussion

The results of the present work revealed that cryptic seed abortion occurred early in pod ontogeny in both beach pea and grass pea. As reported by Nakamura and Stanton (1987) in wild radish, maximum seed abortion in both crops occurred before most of the gain in pod and seed dry mass. This indicates that the maternal plant shows a differential allocation of resources at different times. The seeds that obtained little of the maternal plant's allocation aborted in due course, whereas those seeds that obtained sufficient

maternal allocation developed into normal, healthy seeds (Gurusamy, 1999b). Seed abortion in this manner during various growth stages of pod development enhances maternal fecundity through resource conservation (Nakamura, 1986). Wiens et al. (1987) found reductions in seed-to-ovule ratios of up to 70% to be due to environmental stress in some inbreeding species. In the present study, there was no significant reduction in seed-to-ovule ratio at different growth stages in beach pea which might be due to no significant seed abortion at different growth stages. As a consequence of high seed abortion during initial stages, grass pea showed a reduced seed-to-ovule ratio in the first three growth stages as compared to the last three stages.

In both beach pea and grass pea, all ovules in a pod did not develop into mature seeds. Only about 68% and 82% of ovules in beach pea and grass pea respectively, developed into mature seeds. This was mainly due to the abortion of seeds during various stages of development. It may also be due to a lack of pollination and fertilization as reported by Hossaert and Valero (1988) in *Lathyrus latifolius* and *L. sylvestris*.

The patterns of seed abortion and development at different ovule positions were random in beach pea and nonrandom in grass pea. As reasons for a random pattern of seed abortion, higher frequency of selfing, less inbreeding depression, less genetic diversity of pollen, lower competition among potential sires and random pattern of ovule fertilization and maturation within the pod were reported in *L. sylvestris* (Hossaert and Valero, 1988). A similar pattern of seed abortion as the one observed in grass pea has been reported in *Sophora japonica* (O'Donnell and Bawa, 1993). In both *Lathyrus* species studied, the probability of seed abortion was higher in ovule position 1 followed

by position 2. As pointed out by Hossaert and Valero (1988), this may be due to the fact that ovules in these extreme basal positions are fertilized by the last and slowest-growing pollen tubes. This may affect probability of abortion in at least two ways: (1) embryos fertilized by slow-growing gametophytes may be intrinsically less vigorous in competition with their sibs for maternal resources and (2) it may be that those embryos formed first begin secreting growth-stimulating hormones, thus gaining a temporal advantage as sinks in competition with other embryos for limited maternal resources (Hossaert and Valero, 1988). A significant amount of seed abortion at the stylar end in both crops may be due to the greater distance of these distal ovules from maternal resources.

In conclusion, the random pattern of seed abortion and development was identified within a pod at different growth stages in beach pea, whereas in grass pea the pattern was nonrandom.

Table 8. 1. Means (\pm SE) for pod growth and seed development in beach pea at six reproductive growth stages.

Parameters	Reproductive growth stages					
	S1 (N = 10)	S2 (N = 20)	S3 (N = 20)	S4 (N = 20)	S5 (N = 20)	S6 (N = 10)
Pod shell dry weight (mg)	12.35 \pm 0.23 ^f	37.20 \pm 0.28 ^e	72.63 \pm 0.26 ^d	104.44 \pm 0.33 ^c	159.60 \pm 0.31 ^a	124.73 \pm 0.63 ^b
Seed dry weight (mg)	0.20 \pm 0.01 ^e	0.41 \pm 0.01 ^a	1.70 \pm 0.08 ^d	7.84 \pm 0.10 ^c	32.18 \pm 0.10 ^a	31.43 \pm 0.02 ^b
Aborting seeds/pod	2.10 \pm 0.66 ^a	3.30 \pm 0.43 ^a	3.25 \pm 0.35 ^a	3.15 \pm 0.41 ^a	2.70 \pm 0.32 ^a	2.90 \pm 0.57 ^a
Developing seeds/pod	6.60 \pm 0.65 ^a	6.30 \pm 0.46 ^a	6.10 \pm 0.34 ^a	6.35 \pm 0.35 ^a	6.80 \pm 0.33 ^a	6.30 \pm 0.65 ^a
Ovule+seeds/pod*	8.70 \pm 0.34 ^b	9.60 \pm 0.25 ^a	9.35 \pm 0.20 ^{ab}	9.50 \pm 0.17 ^{ab}	9.50 \pm 0.20 ^{ab}	9.20 \pm 0.51 ^{ab}
Seed-to-ovule ratio	0.76 \pm 0.08 ^a	0.66 \pm 0.05 ^a	0.66 \pm 0.04 ^a	0.68 \pm 0.04 ^a	0.71 \pm 0.03 ^a	0.68 \pm 0.06 ^a

* Number of ovules per ovary was not standardized.

Means (\pm SE) in the same row followed by the same letter are not significantly different at $P = 0.05$ using Duncan's multiple comparison test.

Table 8. 2. Means (\pm SE) for pod growth and seed development in grass pea at six reproductive growth stages.

Parameters	Reproductive growth stages					
	S1 (N = 22)	S2 (N = 37)	S3 (N = 58)	S4 (N = 45)	S5 (N = 17)	S6 (N = 16)
Pod shell dry weight (mg)	19.71 \pm 0.21 ^f	54.58 \pm 0.47 ^e	116.48 \pm 0.24 ^d	164.68 \pm 0.19 ^b	190.99 \pm 0.47 ^a	133.04 \pm 0.29 ^c
Seed dry weight (mg)	0.34 \pm 0.01 ^f	3.72 \pm 0.01 ^e	10.81 \pm 0.01 ^d	23.50 \pm 0.17 ^c	34.32 \pm 0.22 ^b	120.68 \pm 0.45 ^a
Aborting seeds/pod	0.68 \pm 0.18 ^b	1.60 \pm 0.13 ^a	1.19 \pm 0.11 ^a	0.64 \pm 0.12 ^b	0.53 \pm 0.19 ^b	0.63 \pm 0.18 ^b
Developing seeds/pod	2.09 \pm 0.21 ^b	1.73 \pm 0.11 ^b	2.12 \pm 0.11 ^b	2.76 \pm 0.12 ^a	3.06 \pm 0.20 ^a	2.81 \pm 0.23 ^a
Ovule+seeds/pod*	2.73 \pm 0.15 ^b	3.30 \pm 0.11 ^a	3.31 \pm 0.07 ^a	3.40 \pm 0.08 ^a	3.59 \pm 0.12 ^a	3.44 \pm 0.13 ^a
Seed-to-ovule ratio	0.77 \pm 0.06 ^{ab}	0.53 \pm 0.03 ^c	0.64 \pm 0.03 ^{bc}	0.82 \pm 0.03 ^a	0.86 \pm 0.05 ^a	0.81 \pm 0.05 ^a

* Number of ovules per ovary was not standardized.

Means (\pm SE) in the same row followed by the same letter are not significantly different at $P = 0.05$ using Duncan's multiple comparison test.

Fig. 8. 1. Beach pea pod collected at S4 showing aborted seeds (short arrows), developing seeds (long arrows), stylar end (se) and basal end (be). Note the aborted seeds (short arrows), which are smaller and yellow in colour.

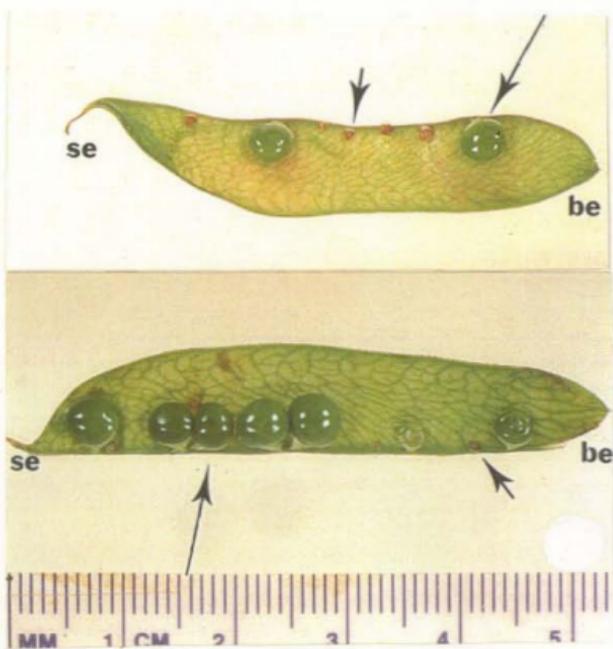


Fig. 8. 2. Frequency distribution of ovules per ovary in beach pea at six reproductive growth stages. Number of ovules per ovary was not standardized. For explanation of stages, see Table 7. 1.

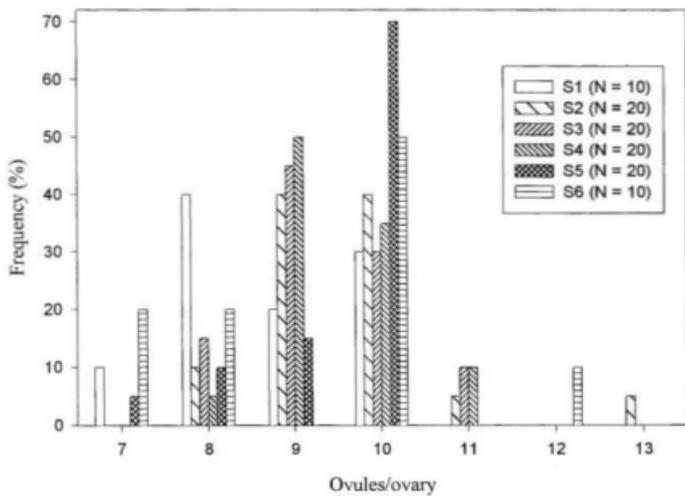


Fig. 8. 3. Shift in the frequency distribution of seeds per pod in beach pea at six reproductive growth stages. Number of ovules per ovary was not standardized. For explanation of stages, see Table 7. 1.

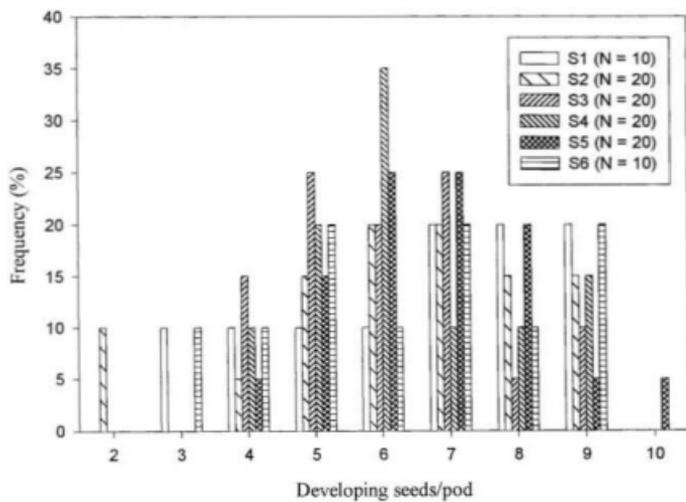


Fig. 8. 4. Frequency distribution of ovules per ovary in grass pea at six reproductive growth stages. Number of ovules per ovary was not standardized. For explanation of stages, see Table 7. 2.

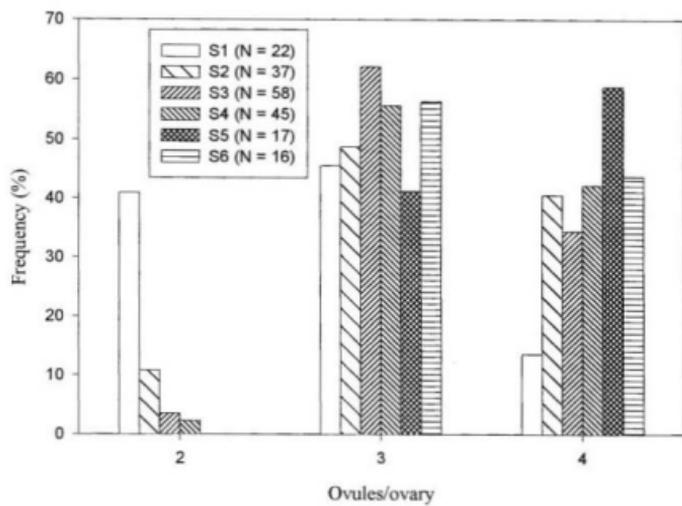


Fig. 8. 5. Shift in the frequency distribution of seeds per pod in grass pea at six reproductive growth stages. Number of ovules per ovary was not standardized. For explanation of stages, see Table 7. 2.

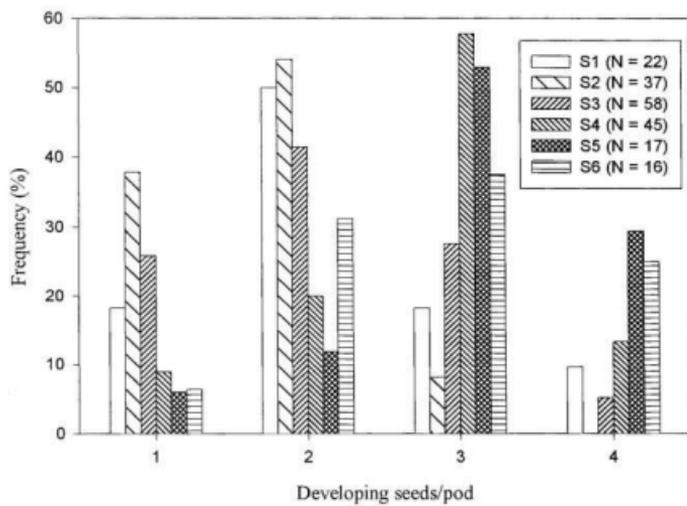


Fig. 8. 6. Probability of seed abortion at different ovule positions within a pod of beach pea at different growth stages. Number of ovules per ovary was standardized as mentioned in materials and methods. For explanation of stages, see Table 7. 1.

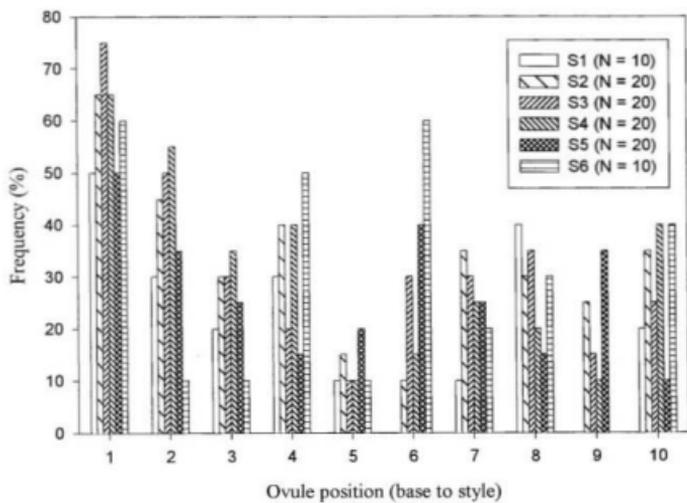


Fig. 8. 7. Probability of seed abortion at different ovule positions within a pod of grass pea at different growth stages. Number of ovules per ovary was standardized as mentioned in materials and methods. For explanation of stages, see Table 7. 2.

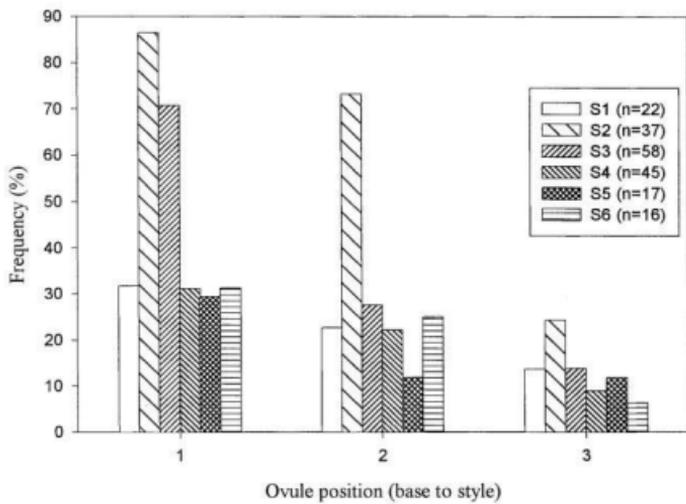


Fig. 8. 8. Probability of seed development at different ovule positions within a pod of beach pea at different growth stages. Number of ovules per ovary was standardized as mentioned in materials and methods. For explanation of stages, see Table 7. 1.

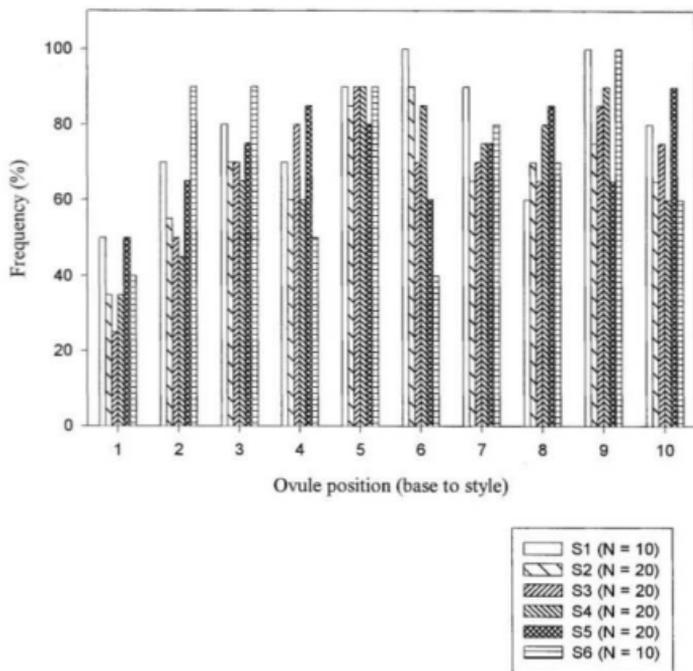
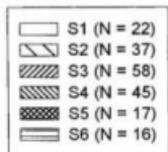
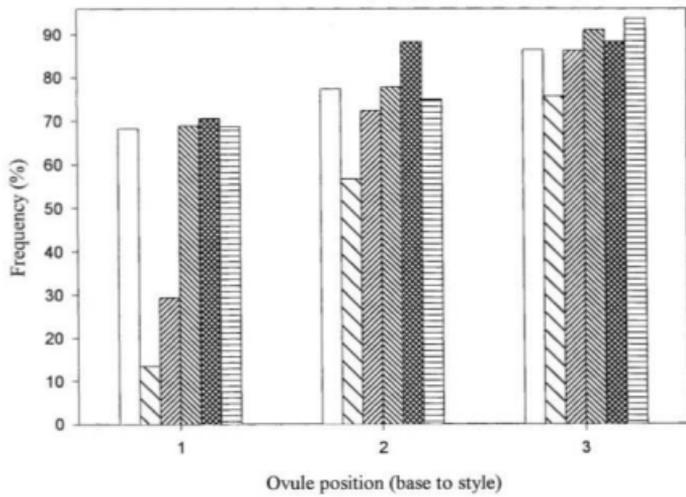


Fig. 8. 9. Probability of seed development at different ovule positions within a pod of grass pea at different growth stages. Number of ovules per ovary was standardized as mentioned in materials and methods. For explanation of stages, see Table 7. 2.



CHAPTER 9

GENERAL CONCLUSIONS

Recent interest in developing a cold-climate crop (McKenzie and Donnelly, 1996) for food, feed or forage in Newfoundland, Canada, has led to studies the circumpolar legume *Lathyrus maritimus* (L.), commonly known as beach pea. For comparison, commercially-available grass pea (*Lathyrus sativus* L.) was also used in some experiments. Seasonal changes in structure and biochemical composition of perennial root nodules collected from natural stands of beach pea at Salmon Cove, Newfoundland, were studied every month for one year. The pattern of seed development, maturation and abortion was also examined.

The native rhizobial strain (ACCCRC) isolated from root nodules of beach pea was the most efficient and effective inoculum when compared with 8 other USDA strains in terms of nodulation and nitrogen-fixing traits (Chapter 2). Perennial root nodules of beach pea undergo winter dormancy and regenerate in the spring. They store large quantities of storage organelles such as oleosomes and amyloplasts prior to winter. The number of oleosomes was higher in winter nodules than in summer nodules (Chapter 3). Accumulation of total lipids also increased towards winter. A microscopical study showed dormant nodule tissue and bacteria in winter without any loss of structural integrity, and some degradation of oleosomes in winter. Peridermal cells of winter nodules were suberized, which is an important protection against cold stress and pathogen attack during winter dormancy.

Oleosomes can store large quantities of energy within the least amount of space (Huang, 1992). The catabolism occurred in oleosomes in winter, which gives multiple benefits: release of energy for metabolism, prevention of freezing of nodule tissue, insulation of cells and a supply of fatty acids for membrane synthesis during regrowth (Chapter 3). Therefore, having a store of oleosomes in the nodule at the end of the growing season is considered to be a winter survival strategy for perennial nodules. Phospholipids and monoglycerides were identified as the major oleosomic lipids of beach pea nodules in all seasons (Chapter 4). C16:0, C18:0 and C18:1 were the dominant fatty acids. In addition to *de novo* synthesis, it seems that nodules selectively utilized some fatty acids to produce others in order to overcome the seasonal influences. The level of many unsaturated fatty acids increased during winter. This could help to maintain the membrane fluidity and function of the cell during winter dormancy.

The level of starch increased in summer and decreased in winter (Chapter 5). This is further verified by microscopical studies, which showed an accumulation of amyloplasts with starch grains in summer and their degradation in winter. Levels of soluble sugars and non-reducing sugars were high in the mid-winter. This could help to maintain high osmolarity in nodule cells to protect them from freezing. Reducing sugars were high in the fall. The results indicate that sugars may possibly serve as cryoprotectants during winter dormancy of nodules.

Protein content of nodules increased from mid-summer to early winter (Chapter 6). The composition of total amino acids in nodules differed widely depending on the season. Arginine, cystathionine, ethanolamine, histidine, hydroxyproline, ornithine and

proline increased in winter, whereas γ -aminobutyric acid decreased in winter. Some of these amino acids could act as osmotic compounds (e.g. proline), protein protector and nitrogen and carbon sources during stress. Environmental conditions and the genetic makeup of plants heavily influence the elemental composition of plants. Higher amounts of P, K, Ca, Mn, Cu and Zn were found in winter nodules, whereas summer nodules had more Cl, Na, S, Mg, Al, Si, Mo and Fe. Most elements are part of essential compounds, which participate in various metabolic processes, therefore, seasonal changes in the elemental composition of nodules could play an important role in nodule life. For example, K is essential for the maintenance of turgor pressure and osmotic potential of cells (Salisbury and Ross, 1986). Thus, high K in winter could protect the nodules from cold stress by preventing cellular dehydration. Like K, every element may play season-specific roles. However, they are not clear at present and need further studies.

The present study suggests, based on microscopical and biochemical evidence, that winter survival of beach pea root nodules is a complex phenomenon which involves a combination of many events such as season-specific adjustments in structural, physiological and biochemical processes of the nodule. It is important to keep in mind that changes in oleosomes, carbohydrates, proteins and elements are not entirely responsible for cellular adaptations to winter stress. It is likely to be influenced by other factors such as genes, hormones and other substances. The genetics behind the winter survival of perennial root nodules, which remains to be studied, will provide further insights.

The studies of seeds revealed that beach pea seeds follow almost a sigmoidal pattern of development (Chapter 7). Beach pea seeds started to show hard seed coat development at S4. Hardseededness increased at S5 and S6, which completely prevented water permeability and, thus, seed germination. In both beach pea and grass pea seeds, S6 was identified as the physiological maturity stage. The study suggests that harvesting of a seed crop at S6 will ensure seeds of high quality. During the course of development, higher seed abortion was observed in beach pea than in grass pea (Chapter 8). Only 68% of the ovules developed into mature seeds in beach pea, while in grass pea it was 82%. The pattern of seed abortion and development at different ovule positions within developing pods was random in beach pea but nonrandom in grass pea. Further studies are needed to more fully assess the seed technology of beach pea. Such studies should be extended from the fundamental to an applied level.

The winter survival capacity of perennial root nodules along with perennial nature of plants, good growth characteristics (McKenzie and Donnelly, 1996), high seed production (Martin et al., 1998c) and the nutritional value of the seeds (Chavan, 1998) suggest that beach pea may be a potential cold-climate crop, which could be developed in future for food, feed or forage.

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