

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Asparagus somatic embryogenesis: detection of somaclonal variation using molecular and cytological analyses

A thesis presented in partial fulfilment of the requirements for the degree

of

Doctor of Philosophy in Horticultural Biotechnology

at

Massey University

Wendy Hollingsworth

1998

Dedicated to:

My parents - Rudolph and Shirley Hollingsworth

“.....because you love me.....”

In pursuit of excellence !!!!!

Abstract

The embryogenic potential for six asparagus cultivars (Aspiring, Karapiro, Pacifica, Turoa, Syn4, and UC157), and the genetic stability of the somatic embryogenic system were investigated. Experiments 1 to 3 investigated the embryogenic potential of select cultivars, whereas experiments 4 to 7 analysed the genetic stability of embryogenic cells and plantlets. In experiment 8, morphological, anatomical, cytological and molecular techniques were used to characterise different types of calli identified during the study.

For all cultivars, embryogenic callus was promoted on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and one of the following plant growth regulator (PGR) concentrations: 0.3, 1, 3, and 10 μM 2,4-D and 1.0 μM NAA/ 0.1 μM Kinetin. Plant genotype, PGR concentration and length of time in culture significantly influenced both the number of explants producing calli and the type of calli developing from explants.

The following sequence was found to be most effective in producing complete plantlets from embryogenic calli: callus induction (CI) on Murashige and Skoog (MS) media containing 3% sucrose, 1% agar and either of 1.0, 3.0 and 10 μM 2,4-D, followed by transfer onto liquid embryo induction media (EI) containing MS + 6% sucrose and finally regeneration on regeneration media (Rg4) containing MS + 0.2 g/l glutamine + 3% sucrose + 1% agar. Treatment of 'Pacifica' globular embryos at -15°C for 3 hr produced the highest percent converted plantlets (34 and 26% for 6-month-old embryogenic calli and 1 year-old embryogenic suspension cells respectively).

The number of *in vitro*-regenerated asparagus plantlets surviving acclimatisation was increased by acclimatising plantlets with minicrowns that contain 2-5 storage roots, and by removal of *in vitro*-formed cladophylls prior to acclimatisation.

Random amplified polymorphic DNA (RAPD) markers distinguished among asparagus cultivars, and revealed differences within seed-raised commercial cultivars. The RAPD

technique also detected changes in genomic DNA structure induced during culture of embryogenic cells. No change in genomic structure of plantlets regenerated from somatic embryos was detected.

Cytological analysis, using chromosome counts and DNA content analysis, were used to determine the genetic stability of embryogenic calli, suspension cells, and plantlets regenerated through somatic embryogenesis. The basic chromosome number of 20 ($2n = 20$) remained unchanged for all samples. The DNA content of explants and plantlets was similar, indicating that plantlets were diploid. The experiment was unable to detect somaclonal variation, revealed by altered ploidy level indicating that cytological analysis is not as sensitive as RAPD analysis for detecting somaclonal variation.

Extracellular protein profiles generated for embryogenic cells grown in suspension culture were influenced by PGR concentration and length of time in culture, and were therefore not suitable for monitoring somaclonal variation.

Overall, individual cultivars produced between 6 to 8 different calli types for all PGR treatments. Plant genotype and PGR treatment influenced the phenotype of calli developed for each cultivar. The results indicate that, for the six asparagus cultivars investigated in this study, nodular calli or nodular mucilaginous calli have more embryogenic potential than other calli types. These calli were also noted to produce embryogenic cells in suspension, and could, therefore, be used to successfully inoculate liquid cultures either for small or large-scale production of asparagus somatic embryos.

Keywords

Asparagus officinalis L., *in vitro*, plant growth regulator, somatic embryos, embryogenic calli, maturation, regeneration, plantlet acclimatisation, random amplified polymorphic DNA (RAPD), chromosome count, DNA content, extracellular protein

Acknowledgements

My heart felt thanks to my supervisors Drs B Christie, M Nichols, and H Behboudian for their excellent supervision and guidance during my research. I especially appreciate being given the independence to set my research pace and direction.

I am grateful to my Government and the NZ Commonwealth Scholarship Committee for awarding me the Commonwealth Scholarship and making it possible for me to complete my Ph.D. study. Thanks also to the NZ Asparagus Council for partially funding my research project. Funding from Massey University Research Fund and the Graduate Research Fund was much appreciated.

I would like to acknowledge the assistance of the following persons during various experiments, Dr D Greer, from HortResearch National Climate Laboratory in Palmerston North, for access to the laboratory facilities and technical assistance during the acclimatisation experiment. Drs P Lockhart (Department of Plant Biology) and G Jonas (Department of Microbiology), and Mr H Neilson (Department of Plant Science) for technical advice and suggestions during molecular analysis. Drs D Harding (Department of Biochemistry), D Cook, and K Wurm (Department of Plant Science) for their useful comments during biochemical analysis experiments. Dr O Campanella and J Latham (Department of Food Technology) for assistance in viscosity determination. Dr A Rowland and E Nickless (Department of Plant Biology) for technical assistance during cytological and confocal microscopic analysis. Thanks also to I O'Brien from the HortResearch Cytoflowametric Laboratory, Auckland for DNA content analysis.

I am also grateful to the following Departments for use, or loan, of equipment: Ecology, Food Technology, Microbiology, and Animal Science.

An appreciation is extended to the team at the photographic unit for photography of electrophoretic gels, to M Alexander for advice with computer graphics, to L Davis for speedy acquisition of materials required for my experiments and to the team at the Plant Growth Unit for maintenance of plant material throughout my project.

I am also grateful to Mr R Thomas for editorial comment and Dr L Opara for general comments on content and style of the thesis. An appreciation is also extended to Professor K Milne and the Secretaries for their ready smiles and continued interest in my progress. To my flatmates (Evelyn, Emma, and David) and the 'Dinner Club' (especially Harumi, Sylvia, and Winny) for helping me maintain a healthy balance between my academic and social life..... Thank you.

Finally, I am eternally grateful to my parents, brother, and sister for their love, support, and confidence in my ability to achieve my goals.

Contents

	Pages
i. Abstract	i
ii. Acknowledgments	iii
iii. Contents	v
iv. List of Tables	xiii
v. List of Figures	xv
vi. List of Plates	xvi
vii. List of Abbreviations	xvii

SECTION 1 ASPARAGUS PRODUCTION

1	General Introduction	1
1.1	Asparagus production	1
1.1.1	Asparagus distribution	1
1.1.2	International asparagus production	1
1.2	Asparagus breeding and production	2
1.2.1	Asparagus genome	2
1.2.2	Conventional asparagus production	3
1.2.3	Non-conventional asparagus production	4
1.2.3.1	Shoot tip culture	4
1.2.3.2	Meristem tip culture	5
1.2.3.3	Adventitious shoot culture	5
1.2.3.4	Protoplast culture	6
1.2.3.5	Haploid cell culture	6
1.2.3.6	Somatic embryogenesis	7
1.3	Asparagus clonal propagation in New Zealand	8
1.4	Research problem, aim and objectives	9
1.4.1	Research problem	9
1.4.2	Research aim	10
1.4.3	Research objectives	10

SECTION 2 SOMATIC EMBRYOGENESIS

2	Somatic Embryogenesis: literature review	11
2.1	Introduction	11
2.1.1	Naturally occurring somatic embryogenesis	11
2.1.2	<i>In vitro</i> somatic embryogenesis	12
2.2	Somatic embryogenic process	13
2.2.1	Induction of embryogenic calli	14

2.2.2	Somatic embryo development	15
2.2.3	Embryo maturation	17
2.2.4	Embryo conversion	19
2.3	Molecular and Biochemical events	20
2.3.1	Biochemical events	21
2.3.1.1	Lipids	21
2.3.1.2	Intra-cellular proteins	22
2.3.1.3	Extra-cellular proteins	23
2.3.1.4	Carbohydrates	24
2.3.2	Molecular events	24
2.4	Summary of objectives for section 2	25

Asparagus somatic embryogenesis: 1. Induction of embryogenic calli using varying plant growth regulator concentrations

	Abstract	26
3.1	Experiment 1	27
3.1.1	Introduction	27
3.1.2	Materials and methods	28
3.1.2.1	Plant material	28
3.1.2.2	Callus induction	29
3.1.2.3	Statistical analysis	29
3.1.3.4	Colour chart	29
3.1.3	Results	30
3.1.3.1	Callus induction	30
3.1.3.2	Condition of explant	31
3.1.3.3	Selection of embryogenic calli	31
3.1.4	Discussion	38
3.1.5	Summary	39

Asparagus somatic embryogenesis: 2. Somatic embryo development, maturation and regeneration

	Abstract	40
3.2	Experiment 2	41
3.2.1	Introduction	41
3.2.2	Materials and Methods	42
3.2.2.1	Plant material	42
3.2.2.2	Somatic embryo induction and maintenance of embryogenic calli	42
3.2.2.3	Growth of embryogenic cells	43
3.2.2.4	Somatic embryo development	43
3.2.2.5	Somatic embryo maturation	43
3.2.2.6	Embryo regeneration	44
3.2.2.7	Maintenance of plantlets regenerated from somatic embryos	44

3.2.2.8	Shock-treatment of long-term embryogenic suspension cells	45
3.2.2.9	Temperature treatment of globular somatic embryos	45
3.2.3	Results	45
3.2.3.1	Somatic embryo development from embryogenic suspensions	45
3.2.3.2	Growth of embryogenic cells in suspension	46
3.2.3.3	Long-term embryogenic cells	47
3.2.3.4	Somatic embryo maturation	48
3.2.3.5	Embryo regeneration	48
3.2.3.6	Temperature effect on embryo conversion	49
3.2.3.7	Abnormal physiological development of somatic embryos	55
3.2.4	Discussion	62
3.2.5	Summary	63

Acclimatisation of plantlets regenerated from asparagus somatic embryos

	Abstract	65
3.3	Experiment 3	67
3.3.1	Introduction	67
3.3.1.1	Acclimatisation of <i>in vitro</i> -produced plantlets: an overview	67
3.3.1.2	Acclimatisation of <i>in vitro</i> -regenerated asparagus plantlets	69
3.3.2	Material and methods	70
3.3.2.1	<i>In vitro</i> -regenerated plantlets	70
3.3.2.2	Plantlet acclimatisation	70
3.3.2.3	Acclimatisation with or without <i>in vitro</i> -developed cladophylls	71
3.3.2.4	Seedlings	71
3.3.2.5	Photosynthesis and transpiration	71
3.3.2.6	Statistical analysis	72
3.3.3	Results	73
3.3.3.1	Acclimatisation of plantlets	73
3.3.3.2	Acclimatisation with or with out <i>in vitro</i> -developed cladophylls	73
3.3.3.3	Photosynthetic capacity of plantlets	74
3.3.3.4	Growth of plantlets in the greenhouse	75
3.3.4	Discussion	81
3.3.5	Summary	83
3.3.6	Acknowledgments	84

SECTION 3 GENETIC ANALYSIS

4	Variability Within <i>In vitro</i>-Cultures Plant Cells:	
	literature review	85
4.1	Introduction	85
4.2	Variation in vegetatively propagated plants	85
4.2.1	Plant sports	86
4.2.2	Chimeras	86
4.2.3	Variegation	87
4.3	Causes of genetic change in plant genomes	87
4.3.1	Reproductive mechanisms	88
4.3.2	Repeat sequences	88
4.3.3	Movement of DNA between organelles	89
4.3.4	Presence of infectious organisms	89
4.3.5	Genetic engineering	90
4.3.6	Epigenetic effects	90
4.4	<i>In vitro</i>-associated genetic variation	91
4.4.1	Somaclonal variation	91
4.4.2	Factors influencing <i>in vitro</i> genetic variation	92
4.4.2.1	Ploidy level and genotype	93
4.4.2.2	Explant source and age	93
4.4.2.3	Culture environment	93
4.4.2.4	Length of time in culture	94
4.4.2.5	Pattern of growth and mode of regeneration	94
4.5	Detecting genetic variability in regenerants	95
4.5.1	Phenotypic analysis	96
4.5.1.1	Morphological characteristics	96
4.5.1.2	Biochemical analysis	96
4.5.1.3	Protein electrophoretic analysis	96
4.5.1.4	Secondary product analysis	97
4.5.2	Genetic analysis	97
4.5.2.1	Cytological analysis	97
4.5.2.2	Molecular analysis	98
4.5.3	Molecular techniques useful for detecting somaclonal variation	99
4.5.3.1	Restriction fragment length polymorphism (RFLP)	99
4.5.3.2	Polymerase chain reaction (PCR)-based techniques	100
4.6	Summary of objectives for section 3	103

Detection of variation among and within asparagus hybrids using random amplified DNA (RAPD) markers

	Abstract	104
5.1	Experiment 4	105
5.1.1	Introduction	106
5.1.2	Materials and methods	106
5.1.2.1	Plant material	106
5.1.2.2	DNA extraction	106
5.1.2.3	RAPD procedure	107
5.1.2.4	Improved resolution of amplified bands	108
5.1.2.5	RAPD analysis of seedlings	108
5.1.3	Results	108
5.1.3.1	Screening explants with primers	108
5.1.3.2	Polymorphisms produced by primers	109
5.1.3.3	Improved resolution of amplified products	109
5.1.3.4	Reproducibility of DNA fingerprints	112
5.1.3.5	Cultivar comparison and variation within cultivars	112
5.1.4	Discussion	116
5.1.5	Summary	117
5.1.6	Acknowledgments	117

Variation within asparagus embryogenic calli, suspension cells and plantlets regenerated from somatic embryos detected using RAPD markers

	Abstract	118
5.2	Experiment 5	120
5.2.1	Introduction	120
5.2.2	Materials and methods	120
5.2.2.1	Plant material and culture conditions	120
5.2.2.2	DNA extraction	121
5.2.2.3	RAPD procedure	122
5.2.2.4	Gel electrophoresis and staining	122
5.2.2.5	Nomenclature	122
5.2.3	Results	122
5.2.3.1	DNA yields	122
5.2.3.2	RAPD analysis of explants	122
5.2.3.3	Primer effectiveness to reveal polymorphisms	123
5.2.3.4	Specific polymorphisms revealed for each cultivar	124
5.2.3.5	Effect of PGR treatment and length of time in culture on genetic stability of cultivars	133
5.2.4	Discussion	139
5.2.5	Summary	142

5.2.6	Acknowledgments	142
-------	-----------------	-----

Cytological analysis of asparagus embryogenic calli, suspension cells and regenerated plantlets

	Abstract	143
5.3	Experiment 6	144
5.3.1	Introduction	144
5.3.2	Materials and methods	146
5.3.2.1	Donor plant material	146
5.3.2.2	Calli and embryogenic cells	146
5.3.2.3	Regenerated plantlets	146
5.3.2.4	Determination of ploidy level	147
5.3.2.5	Determination of nuclear DNA content	147
5.3.2.6	Flow cytometry	148
5.3.3	Results	149
5.3.3.1	Chromosome counts	149
5.3.3.2	DNA content	149
5.3.4	Discussion	151
5.3.5	Summary	152
5.3.6	Acknowledgments	152

Detection of extracellular proteins secreted by asparagus embryogenic cells in suspension cultures

	Abstract	153
5.4	Experiment 7	154
5.4.1	Introduction	154
5.4.2	Materials and methods	155
5.4.2.1	Plant material	155
5.4.2.2	Embryogenic cell culture	155
5.4.2.3	Extracellular protein extraction	156
5.4.2.4	Protein content determination	156
5.4.2.5	Extracellular glycoprotein detection	157
5.4.3	Results	157
5.4.3.1	Cultivar specific extracellular proteins	157
5.4.3.2	PGR treatment effect on extracellular protein profiles	158
5.4.3.3	Stability of extracellular protein profiles over time	161
5.4.3.4	Protein profiles of cells grown in medium with or with out PGRs	161
5.4.3.5	Extracellular glycoproteins	162
5.4.4	Discussion	165
5.4.5	Summary	166
5.4.6	Acknowledgments	167

SECTION 4 CALLUS CHARACTERISATION

Characterisation of long-term asparagus calli maintained on medium containing plant growth regulators

	Abstract	168
6.1	Callus characterisation: a synthesis of techniques	170
6.1.1	Introduction	170
6.1.2	Materials and methods	172
6.1.2.1	Plant material	172
6.1.2.2	Callus tissue	172
6.1.2.3	Callus phenotype	172
6.1.2.4	Type of cell suspension obtained on PGR-free medium	172
6.1.2.5	Preparation of samples for confocal microscopy –	173
6.1.2.6	Viscosity of culture medium	174
6.1.2.7	Embryo maturation and regeneration	174
6.1.2.8	RAPD analysis of calli	174
6.1.2.9	Cytological analysis	175
6.1.3	Results	175
6.1.3.1	Callus phenotype	175
6.1.3.2	Characterisation of cell suspensions	175
6.1.3.3	Embryo maturation and regeneration	177
6.1.3.4	RAPD analysis of calli	177
6.1.3.5	Cytological analysis of calli	178
6.1.4	Discussion	191
6.1.5	Summary	194
6.1.6	Acknowledgments	195

General discussion, conclusions and recommendations

7.1	General discussion and conclusions	196
7.2	Research application	200
7.2.1	Direct application	200
7.2.2	Future application	201
7.3	Recommendation for future research	203
7.3.1	Embryo maturation	203
7.3.2	Genetic stability	204
7.3.3	Bioreactor production	204
8	REFERENCES	205
	APPENDICES	227

List of Tables

	Page
Table 1.1 International asparagus production	2
Table 3.1 Effect of PGR treatment on the number of explants producing callus	33
Table 3.2 Morphology of explants grown on callus induction medium	35
Table 3.3 Description of callus phenotype	36
Table 3.4 Somatic embryo development and morphology on induction medium	47
Table 3.5 Regeneration of somatic embryos	49
Table 3.6 Plantlet generation from cultivar Aspiring globular embryos	50
Table 3.7 Survival of acclimatised <i>in vitro</i> -developed 'Pacifica' plantlets	76
Table 3.8 Effect of plantlet storage roots on acclimatisation and survival after 5 weeks in the greenhouse	76
Table 5.1 Asparagus cultivars evaluated by RAPD analysis	107
Table 5.2 Polymorphic bands of asparagus cultivars	115
Table 5.3 List of primers and the number of DNA bands amplified for each cultivar	125
Table 5.4 Cultivar specific polymorphic markers useful for identifying 'Aspiring', 'Karapiro', and 'Pacifica'	126
Table 5.5 Total number of variant DNA profiles revealed by all primers for asparagus cultivars Aspiring, Karapiro, and Pacifica	129
Table 5.6 Variant DNA bands observed for asparagus embryogenic cells grown on PGR-free liquid medium for one year	130
Table 5.7 Variant polymorphic bands observed for samples of asparagus embryogenic calli maintained on PGR medium for one year	134
Table 5.8 Variant DNA profiles revealed for asparagus cultivars Aspiring, Karapiro, and Pacifica by all primers for embryogenic calli and suspension cells	137
Table 6.1 Calli types developed for asparagus cultivars grown on PGR medium	179
Table 6.2 Type of calli developed for asparagus cultivars grown on PGR-free medium	181
Table 6.3 Phenotype of globular embryos produced for asparagus cultivars grown on embryo induction medium and then transferred to regeneration medium	185
Table 6.4 Plantlets regenerated from cultivars Aspiring and Turoa globular and mature somatic embryos derived from various calli types	187
Table 6.5 Conversion of 'Aspiring' globular embryos derived from calli type E	187
Table 6.6 Polymorphic DNA bands detected for calli maintained on callus induction medium	188

Appendices

Table 5.0	DNA profiles generated for six asparagus cultivars using 20 10-base primers	228
-----------	--	-----

List of Figures

	Page
Figure 3.1 Plant growth regulator treatment effect on the number of explants producing calli _____	34
Figure 3.2 Growth of cultivars Aspiring, Karapiro, and Pacifica embryogenic cells in embryo induction medium _____	53
Figure 3.3 Growth of asparagus embryogenic cells in embryo induction medium _____	54
Figure 3.4 Photosynthesis and transpiration of 5-week-old acclimatised 'Pacifica' plantlets and 5-week-old 'Syn4' seedlings _____	78
Figure 5.1 Extracellular protein profiles of asparagus cultivars Aspiring, Karapiro, and Turoa _____	159
Figure 5.2 Type and concentration of PGR effect on extracellular protein profiles of asparagus embryogenic cultures _____	160
Figure 5.3 Time effect on extracellular protein expression of cultivar Aspiring embryogenic cultures _____	163
Figure 5.4 PGR effect on expression of putative embryogenic-associated proteins secreted by cultivar Aspiring embryogenic cells _____	164
Figure 6.1 Viscosity of culture medium 4 weeks after growth of calli on PGR-free medium _____	183
Figure 7.1 Production of asparagus synthetic seed _____	202

List of Plates

	Pages
Plate 3.1	Growth of explants on callus induction medium _____ 37
Plate 3.2	Somatic embryo development on EI1 medium _____ 51
Plate 3.3	Somatic embryos _____ 52
Plate 3.4	Somatic embryo aggregates _____ 56
Plate 3.5a	Somatic embryo development _____ 57
Plate 3.5b	Somatic embryo development _____ 58
Plate 3.6	Precocious germination of somatic embryos _____ 59
Plate 3.7	Recurrent embryogenesis _____ 60
Plate 3.8	Rhizogenesis and organogenesis _____ 61
Plate 3.9	<i>In vitro</i> root development on cultivar Pacifica plantlets regenerated from somatic embryos _____ 77
Plate 3.10	Asparagus plant _____ 79
Plate 3.11	Morphology of 3-month-old greenhouse acclimatised plantlets _____ 80
Plate 5.1	Random amplified polymorphic DNA profiles for PC_9 and SN_7 generated by 10 different primers _____ 110
Plate 5.2	Relative sensitivity of ethidium bromide and silver staining of DNA bands _____ 111
Plate 5.3	Consistency of DNA fingerprints from cultivar Pacifica generated using OPC-12 _____ 114
Plate 5.4	DNA profiles of 'Pacifica' plantlets regenerated from somatic embryos derived from calli initiated on medium containing 10 μ M 2,4-D _____ 132
Plate 5.5	Variation in DNA profiles of 'Pacifica' revealed by different primers _____ 138
Plate 5.6	Asparagus chromosomes (2n=20) _____ 150
Plate 6.1	Phenological characteristics of calli types _____ 180
Plate 6.2	Cell differentiation in somatic embryo suspension cultures _____ 184
Plate 6.3	Confocal laser scanning microscopic images of somatic embryos _____ 186
Appendices	
Plate 3.0	Colour chart _____ 227
Plate 5.7	Extracellular protein profiles of asparagus cultivars _____ 238
Plate 5.8	Extracellular glycoprotein profiles of asparagus cultivars _____ 239

List of Abbreviations

µg	microgram (s)
µl	microlitre (s)
µm	micrometer (s)
µM	micromolar (s)
µmol	micromole (s)
2,4-D	2,4-dichlorophenoxyacetic acid
2C	nuclear DNA content of unreplicated diploid chromosome complement
ABA	Absciscic acid
AFLP	Amplified fragment length polymorphism
ASP	Asparagus cultivar Aspiring
BA (BAP)	Benzylamino purine
CFLP	Cleavase fragment length polymorphism
Chl-	Chlorophyll deficient embryos
Chl+	Globular embryos containing chlorophyll
CI	Callus induction
CRD	Complete random design
DNA	Deoxyribonucleic acid
EI	Embryo induction
g	grams
GA ₃	Gibberellic acid
hr	hour (s)
IAA	3-indole acetic acid
IEDC	Induced embryogenic determined cell
kD	kilodalton
kg	kilogram (s)
Kn	Kinetin
KP	Asparagus cultivar Karapiro
l or L	litres
LEA	Late embryogenesis protein
mg	milligram (s)
min	minute (s)
mm	millimetre (s)
MS	Murashige and Skoog
MW	molecular weight
NAA	α-Napthaleneacetic acid
°C	degrees Celsius
PC	Asparagus cultivar Pacifica
PCR	Polymerase chain reaction
PEDCs	Pre-embryogenic determined cell
PEG	Polyethylene glycol
PEMs	Proembryogenic masses
PGR	Plant growth regulator (s)
PI	Propidium iodide
Pur	Globular embryos with purple pigment
RAPD	Random amplified polymorphic DNA

RFLP	Restriction fragment length polymorphism
Rg	Regeneration
SEM	Standard error of mean
SN	Asparagus cultivar Syn4
SSCP	Single stranded conformation polymorphism
TU	Asparagus cultivar Turoa
UC	Asparagus cultivar UC157

SECTION 1

ASPARAGUS PRODUCTION

