Scientific Research Publishing

American Journal of Plant Sciences, 2021, 12, 603-613
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Influence of Amino Acids on the Secondary Somatic Embryogenesis Proliferation Process of Moroccan Cork Oak (*Quercus suber* L.)

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How to cite this paper: Ben Ali, N. and Ahmad, L. (2021) Influence of Amino Acids on the Secondary Somatic Embryogenesis Proliferation Process of Moroccan Cork Oak (*Quercus suber* L.). *American Journal of Plant Sciences*, **12**, 603-613.
https://doi.org/10.4236/ajps.2021.124040

Received: March 4, 2021
Accepted: April 19, 2021
Published: April 22, 2021

Abstract

The present study aims to explore the regeneration potential of Moroccan cork oak through the secondary somatic embryogenesis process. Particularly, we focus on the analysis of amino acids influence on the quantity and quality of the regenerated secondary embryos. The amino acids tested are: Glutamine, asparagine, arginine, tryptophane, methionine, casein hydrolysate and urea. Each amino acid is added in the Margara (N30K) medium at different concentrations ranged between 10 and 500 mg/l. The results are collected after 2 months of culture. First analysis shows that the glutamine and the casein hydrolysate gives a maximum number of somatic embryos, clusters and pre-embryos newly formed on the clusters. By comparison to the control medium, the increase of the secondary embryos number directly formed exceed 36% in the case of casein hydrolysate and 35% of in the case of glutamine both at the concentration of 30 mg/l. However, the test of the combination of these amino acids did not have any significant results. In terms of quality, the influence of amino acids on the morphology of secondary embryos was analyzed.

Keywords

Quercus suber L., Secondary Embryos, Amino Acids, Glutamine, Asparagine, Casein Hydrolysat

1. Introduction

Cork oak (*Quercus suber* L.) is considered one of the most important multipurpose species in all the Mediterranean area due to its ecological value as an environmental protector. Seed production and cork-derived industries have a major economic input into the maintenance of rural populations [1]. In the Moroccan

cork oak forest ecosystem, the economic interest of this species has facilitated the development of new strategies for reforestation programs [2]. However, conventional breeding of cork oak is constrained by its long reproductive cycle, which includes long juvenile phase, and its complex reproductive biology, including self-incompatibility and a high degree of heterozygosis [3].

However, cloning of adult trees is still difficult, and in some species it is only possible at a juvenile phase because in most woody species, the selection of elite trees is only possible at the adult phase when individuals show their characteristics [4] [5].

The propagation through somatic embryogenesis (SE) is considered to be an important tool for obtaining efficient true to-type vegetative propagation of selected mature trees [6]. Within the genus Quercus, SE induction from mature trees has been reported in many Quercus species like: holm oak [7] cork oak [5], [8], and pedunculate oak [9] [10] [11]. These studies were specifically focused on the embryos multiplication and conversion into plantlets, generally considered to be the most problematical stages limiting the use of SE for mass propagation, due to the low frequency of response [12].

The secondary somatic embryogenesis (SE) is a phenomenon where by new somatic embryos are initiated from other somatic embryos. Some cultures are able to retain their competence for secondary SE for many years, and thus provide useful material for various studies, as described for *Vitis rupestris* [13], cork oak (*Quercus suber*) [4]. Additionally, for some species, primary SE is less efficient than secondary SE, as reported for cork oak [5] [14]. Therefore, in plants with long life cycles, such as dicotyledonous woody plants, preserving embryogenic lines can be a cost-effective maintenance while those lines are being tested in field [15].

One of the most influential factors in the morphogenic response associated with secondary embryos culture is the kind and concentration of growth regulators. Amino acids serve as primary sources of organic nitrogen for the growth of many cells. They additionally promote communication between cells and tissues within multicellular organisms [16]. Organic nitrogen nutrition was also reported to affect somatic embryogenesis in conifers [7]. They have generally been used in the different phases of conifer somatic embryogenesis [17]. Glutamine and other aminoacids, can improve cell proliferation as well as regeneration in specific genotypes [18] [19] [20] [21].

In this context, the objective of the present work is to study the effect of different sources, levels and balances of nitrogen on both induction and development of secondary SE from mature somatic embryos of Moroccan cork oak (*Quercus suber* L.).

Our work has been also devoted to study for the first time the secondary somatic embryogenesis process of Moroccan cork oak *Quercus suber* L. The objective was to explore the embryogenic potential through the secondary somatic embryogenesis process. This process is considered as an effective means for the mass propagation and maintenance of somatic embryos for micropropagation and

its application to Moroccan cork oak. Mainly, we focus on the influence of amino acids on proliferation secondary somatic embryos.

2. Material and Methods

Mature embryos at the cotyledonal stage, of about 8 - 10 mm in size and approximate fresh weight of about 60 mg from somatic origin, were cultured in 9 cm diameter Petri dishes containing 20 ml of proliferation medium (N30K) and supplemented with different amino acids: glutamine (Gln), arginine (Arg), asparagine (Asn), méthionine (Met), tryptophan (Try), urea (Urea) and casein hydrolysate (CH) or a mixture of the All amino acids combinations. The experiment was arranged in a completely randomized design with 28 treatments. All cultures were maintained in a constant ambient temperature of 25˚C ± 2˚C in darkness.

For this experiment, 30 explants somatic embryos were cultured per experimental unit in each of the 28 treatments (the control and seven amino acid treatments). All the somatic embryos were homogeneously distributed between treatments. The experiment was repeated three times, thus a total of 840 embryos was used.

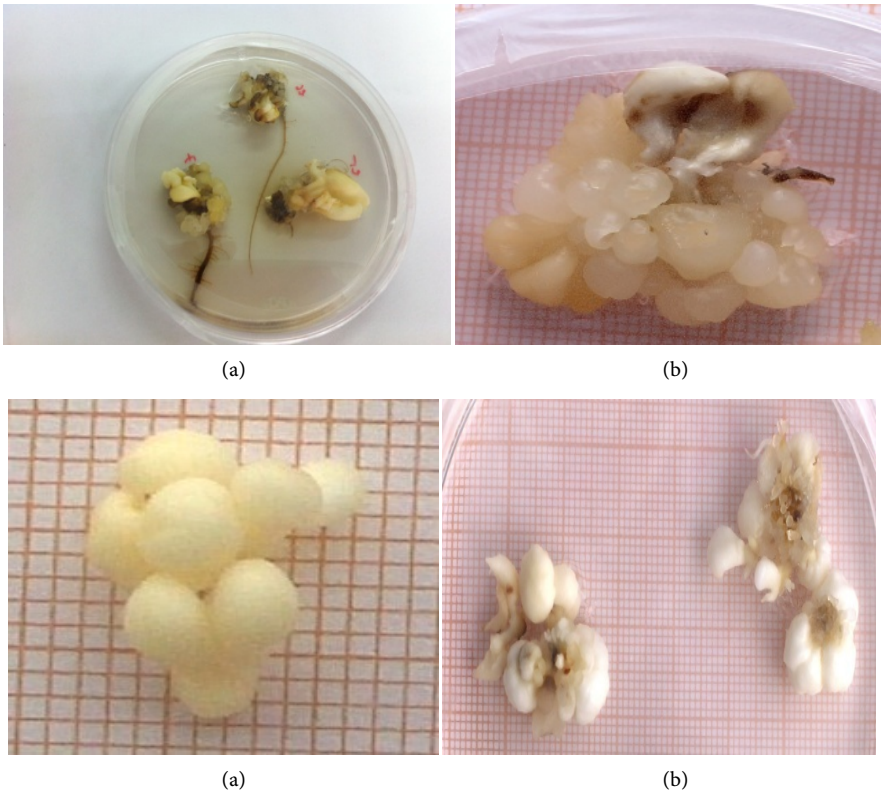
Percentage of explants with secondary SE and number of viable clusters and somatic embryos on clusters per explants (primary somatic embryo) were recorded after 8 weeks in culture. The resulting data were submitted to a statistical package SPSS 17.0. [22]. One-way analysis of variance (ANOVA) was carried out to determine the differences between the treatments that produced cotyledonal somatic embryos. Multiple comparisons were made using Duncans post-hoc test (a = 0.05).

3. Results and Discussions

The effect of amino acids concentration on ES proliferation is shown in **Table 1**. The control medium is used as reference state. The results are collected after 8 weeks from the initiation of the culture and are presented in terms of average number of SSE formed per primary embryos, average number of clusters per primary embryos and average number of SSE newly formed on clusters.

In general, amino acids show a quite acceptable embryogenic response will compared to the control medium. A visual examination of secondary somatic embryos formed in the presence of Glu or HC shows a translucent aspect with normal morphology. **Figure 1** illustrates some examples of embryos morphology obtained in the presence of amino acids. The increase in the number of secondary embryos exceeds 36% and 35% respectively in the case of casein hydrolyzate and glutamine respectively at the concentration of 30 mg/l (**Figure 2**).

The response of the secondary somatic embryogenesis process differs depending on the type of amino acid used. The highest proliferation ratio of secondary somatic embryos (SSE) was obtained with the 30 mg/l of glutamine and 30 mg/l of CH respectively at 6.16 ± 0.48 and 6.2 ± 0.62 respectively (**Table 1**). In



(a) (b)

(a) (b)

Figure 1. Influence of amino acids on the induction of secondary somatic embryogenesis after 2 months of culture: (a) Regeneration of secondary somatic embryos (SSE) on the control medium; (b) Secondary embryos have a translucent appearance during development; (c) Structure and size of secondary somatic embryos isolated after two months of growth; (d) Effect of Glu at high concentration.

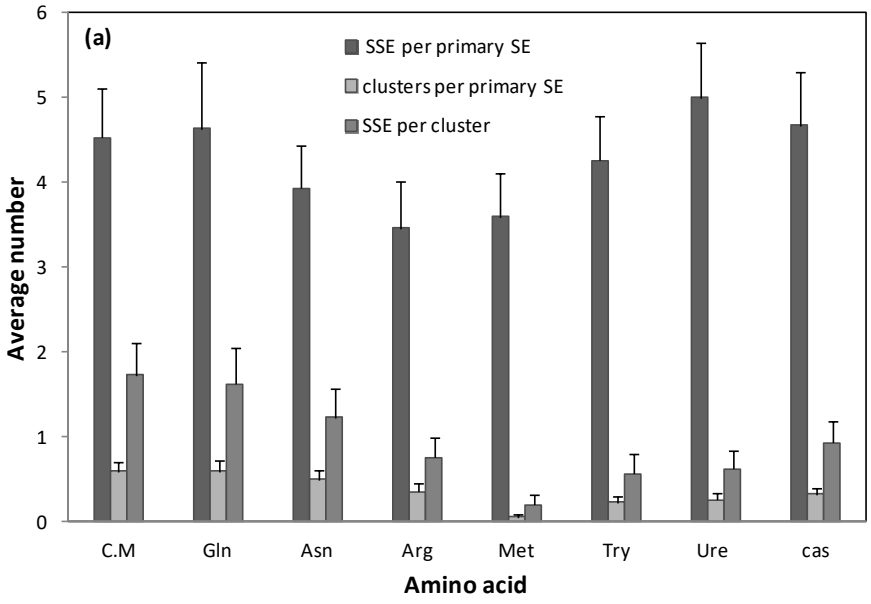
Table 1. Effect of treatment with different concentrations of amino acids on the secondary somatic embryogenesis process of Moroccan *Quercus suber* L.

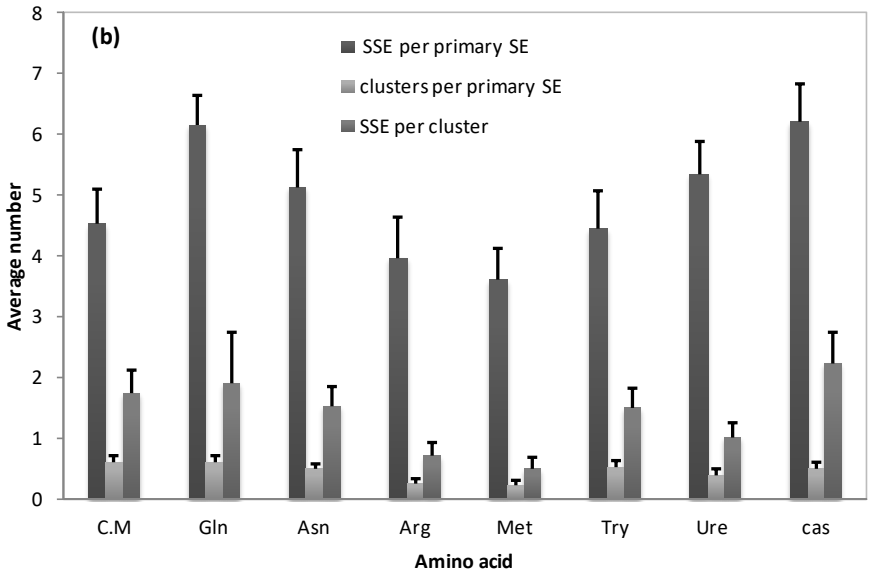
amino acid	Concentration (mg/L)	Average number of SSE per primary SE	Average number of clusters per primary SE	Average number of SSE per cluster
Control medium	0	4.53 ± 0.57 abcde	0.6 ± 0.11 a	1.73 ± 0.39 abc
	10	4.63 ± 0.78 abcde	0.6 ± 0.12 a	1.63 ± 0.43 abcd
Glutamine	30	6.16 ± 0.48 ab	0.6 ± 0.11 a	1.9 ± 0.83 ab
	500	5.26 ± 0.75 abc	0.6 ± 0.13 a	1.67 ± 0.35 abcd
	10	3.93 ± 0.51 cde	0.5 ± 0.11 ab	1.23 ± 0.34 bcdefg
Asparagine	30	5.13 ± 0.63 abcd	0.5 ± 0.09 ab	1.53 ± 0.33 abcdef
	500	5.3 ± 0.62 abc	0.36 ± 0.10 abcd	1.16 ± 0.36 bcdefg
	10	3.46 ± 0.55 cdef	0.36 ± 0.10 abcd	0.76 ± 0.24 cdefg
Arginine	30	3.97 ± 0.66 cde	0.26 ± 0.08 bcd	0.7 ± 0.24 cdefg
	500	3.47 ± 0.63 cdef	0.3 ± 0.08 abcd	0.8 ± 0.26 cdefg
	10	3.6 ± 0.51 cde	0.06 ± 0.04 d	0.2 ± 0.13 f
Methionine	30	3.6 ± 0.52 cde	0.23 ± 0.07 bcd	0.5 ± 0.18 ef
	500	1.83 ± 0.31 fg	0.26 ± 0.08 bcd	0.4 ± 0.13 f

Continued

	10	4.26 ± 0.52 cde	0.23 ± 0.07 bcd	0.57 ± 0.23 ef
Tryptophan	30	4.46 ± 0.62 abcde	0.53 ± 0.09 ab	1.5 ± 0.31 abcde
	500	0.4 ± 0.21 g	0.13 ± 0.06 cd	0.26 ± 0.17 f
	10	5.00 ± 0.64 abcd	0.26 ± 0.08 bcd	0.63 ± 0.22 def
Urea	30	5.33 ± 0.54 abc	0.4 ± 0.09 ab	1.00 ± 0.25 bcdef
	500	3.06 ± 0.44 ef	0.3 ± 0.08 abcd	0.8 ± 0.23 cdef
	10	4.67 ± 0.64 abcde	0.33 ± 0.08 abcd	0.93 ± 0.26 bcdef
Casein Hydrolysat	30	6.2 ± 0.62 a	0.5 ± 0.10 ab	2.23 ± 0.51 a
	500	4.00 ± 0.45 cde	0.29 ± 0.07 abcd	1.61 ± 1.12 ef

Data were recorded after 8 weeks of culture, values are mean ± SE for three repeated experiments, each with three replicate Petri dishes and 3 embryogenic explants per plate. Within each embryogenic line and column, values with the same letter were not significantly different at $P \leq 0.05$ (LSD test).





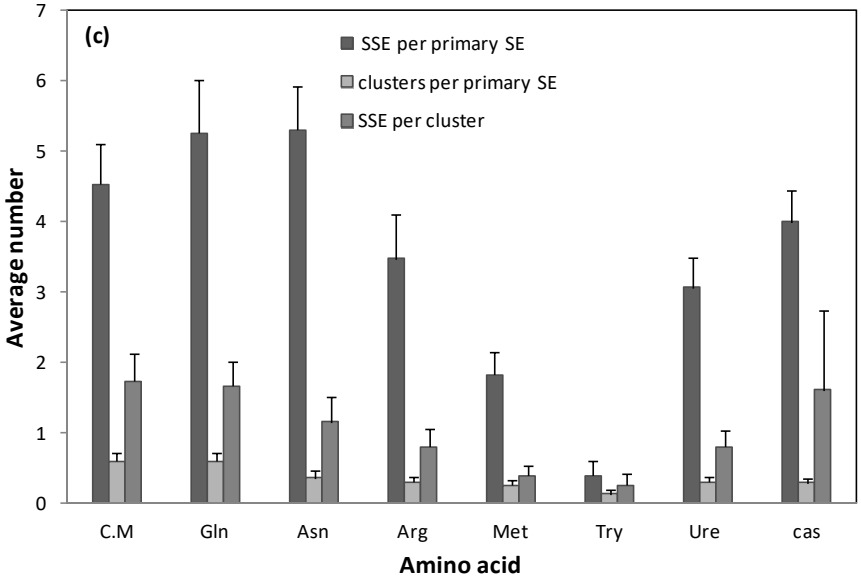


Figure 2. Effect of treatment with different concentrations of amino acids on the secondary somatic embryogenesis process of Moroccan *Quercus suber* L: (a) At concentration of 10 mg/l; (b) At concentration of 30 mg/l; (c) At concentration of 500 mg/l.

addition, clusters and secondary somatic embryos formed on the clusters had a similar proliferation ratio (0.6 ± 0.11 and 1.63 ± 0.83 respectively for clusters and SSE on clusters with Glu; 0.5 ± 0.10 and 1.67 ± 0.35 respectively for clusters and SSE on clusters with CH. It should be noted that the Glutamine is assimilated more rapidly into carbon skeletons of proteins than other inorganic nitrogen sources, and it is known to be an important amino acid in somatic embryogenesis of other species [18].

However, a lower proliferation ratio was obtained on the culture medium containing metheonine with a number of SSE that can not exceed 3.6 ± 0.52 followed by the arginine significant difference and the results remain in general quite acceptable.

On the other hand, results shows that the combinations of the different type of amino acid have not been satisfactory and the results remain without significance even if it exceeds sometimes the ratio of proliferation obtained in the case of the control medium (**Table 2**). In certain cases, we can observe that the induction of secondary somatic embryogenesis decrease significantly when the proliferation medium was supplemented with the combination of the aminoacids (0.9 ± 0.18) (**Figure 3**).

The reported results show that the amino acids in general promote the multiplication and growth of embryos; this may be explained by the fact that these amino acids replace partially the NH4. Furthermore, they increase the levels of reduced nitrogen which stimulates the development of somatic embryos. Usually, the best embryogenic response can be obtained when the amino acids are added to the proliferation medium at moderated concentration (30 mg/l).

Embryo growth is a critical step for the development in vitro and germination

Table 2. Effect of combination of different amino acids on the secondary somatic embryogenesis process of Moroccan *Quercus suber* L.

Combination of amino acid	Concentration (mg/L)	Average number of SSE per primary SE	Average number of clusters per primary SE	Average number of SSE per cluster
Control medium	0	4.53 ± 0.57 abcde	0.6 ± 0.11 a	1.73 ± 0.39 abc
COMB1: Glutamine + Asparagine + Arginine	10 + 10 + 10	3.33 ± 0.42 def	0.36 ± 0.11 abcd	1.46 ± 0.51 abcde
	30 + 30 + 30	3.63 ± 0.39 cde	0.23 ± 0.07 bcd	0.57 ± 0.22 ef
	500 + 500 + 500	4.36 ± 0.48 bcde	0.36 ± 0.08 abcd	0.8 ± 0.25 cdef
COMB2: Glutamine + Asparagine + Arginine + Methionine + Tryptophan	10 + 10 + 10 + 10 + 10	4.43 ± 0.39 abcde	0.6 ± 0.09 a	1.53 ± 0.28 abcde
	30 + 30 + 30 + 30 + 30	5.00 ± 0.46 abcd	0.4 ± 0.09 abc	1.06 ± 0.28 bcdef
	500 + 500 + 500 + 500 + 500	0.9 ± 0.18 g	0.4 ± 0.09 abc	0.9 ± 0.25 bcdef

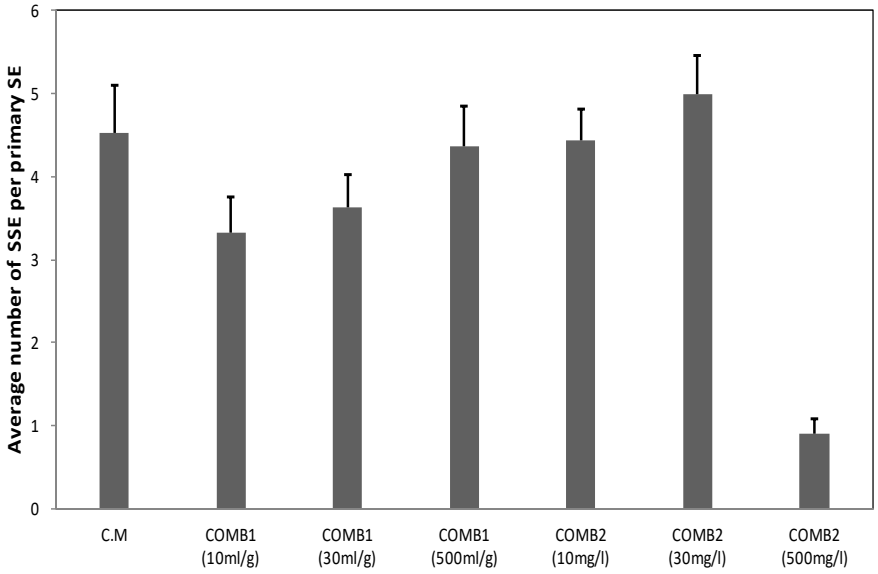


Figure 3. Effect of combination of different amino acids on the secondary somatic embryogenesis process of Moroccan *Quercus suber* L. CM: control medium, COMB1: Glutamine + Asparagine + Arginine, COMB2: Glutamine + Asparagine + Arginine + Méthionine + Tryptophane

of somatic embryos [22] [23]. In this work, it is noted that the Amino acids activate the regeneration of the embryos. However, the need for amino acids in the culture medium depends also on the species; the explant or on the desired morphogenic response. Nevertheless, in some cases growth inhibition has been reported [7] [24] [25]. Merkle *et al.* [26] reported that the addition of amino acids to the culture medium might inhibit or promote the SE development and conversion. Von Arnold [27] found a strong negative effect of L-glutamine, L-arginine, and L-asparagine on induction of somatic embryogenesis in P. *abies*. Kirby *et al.* [28] reported that the addition of CH inhibited growth in cell suspension of *Picea menziesii* [19]. Smith [29] [30] supplemented the maturation medium of *Pinus radiata* with a solution of seven amino acids, to improve somatic embryos production and plantlet recovery, and reported significant effects on somatic em-

bryogenesis. In other plant species, such as *Picea glauca*, some amino acids had either a negative or no effect on somatic embryo maturation [31] [32].

Furthermore, our experiments show that the embryogenic response depends also on the type and the nature of the amino acid used. As illustrated in **Figure 2** the arginine and the metheonine show some kind of inhibition against the proliferation of somatic embryos. Consequently, the poor response obtained in the case of the amino acids combination can be attributed in some measure to the presence of the arginine and the metheonine.

4. Conclusions

The obtained results showed that amino acids tend to favor the multiplication and growth of embryos, embryogenic response was quite acceptable. Compared to the control medium, secondary embryos increased beyond 36% in the case of casein hydrolyzate and 35% glutamine at a concentration of 30 mg/l. The lower rate of proliferation is obtained in the case of methionine followed by arginine, both show some kind of inhibition against the proliferation of somatic embryos.

However, tests on the combination of amino acids did not give satisfaction and results remain without significance. The establishment of histological study of secondary ultratsuctural cell and comparison batwing the content of amino acids and others plant hormones may improve the efficiency of Moroccan cork oak micropropagation.

Fund

This research was partially funded by Hassan II Academy of Science and Technology (Morocco) through project named "vegetative propagation of Moroccan cork oak with the technique of somatic embryogenesis".

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

Arg—L-arginine;
Asn—L-asparagine;
CH—Casein hydrolyzate;
Cl—Clusters;
Gln—L-glutamine;
Met—Methionine;
SE—Somatic embryos, somatic embryogenesis;
SSE—Secondary somatic embryos, secondary somatic embryogenesis;
Try—Tryptophan.