Development of the CELLOP optimisation model for plant cell cultivation

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Abstract

A mathematical computer-aided model CELLOP was constructed in which the desirability functions in a three-dimensional experimental design can be used to find the optimal growing conditions for plant cells. CELLOP is intended for the optimisation of 3 to 6 physical, chemical, or biological variables in the cultivation conditions of plant cell cultures. The model was used to optimise the culturing conditions (calcium, inorganic nitrogen, and sucrose concentrations) for coumarin-producing, spontaneously embryogenic cell lines of angelica *Angelica archangelica* L. subsp. *archangelica* and hogs fennel *Peucedanum palustre* (L.) Moench. For *A. archangelica* the overall optimum concentrations were 0.47 mM Ca^{2+}, 5.06 mM NO_3^-, 0.40 mM NH_4^+, and 96.25 mM sucrose. The dry mass was 24.7 % higher and the coumarin content 40.5 % higher than those achieved in the standard 75 % Gamborg B5 medium. For *A. archangelica* the highest embryogenic activity was reached in the media containing 1.25 mM Ca^{2+}. In the case of *P. palustre* the overall optimum concentrations were 1.60 mM Ca^{2+}, 2.84 mM NO_3^-, 0.23 mM NH_4^+, and 85.10 mM sucrose. For *P. palustre* the dry mass production increased by 61.8 % and the coumarin content by 58.1 % compared to the values achieved in the Gamborg B5 medium. For *P. palustre* the highest embryogenic activity was reached in the presence of 50.0 mM NO_3^- and 4.01 mM NH_4^+.

Additional key words: *Angelica archangelica*, desirability function, embryogenic cell lines, *Peucedanum palustre*.

Introduction

Secondary metabolite production can be increased by genetic engineering, but growth medium optimisation is still an important step in the development of a feasible bioprocess (Verpoorte *et al.* 2002). The traditional approach is to determine the effect of a single explanatory variable on the whole system while omitting the interactions of the other nutrient components. This means that optimisation methods have had to be developed that enable approximation of the interactions between several variables in a multivariable system. Minor modification of the widely used standard medias, such as Gamborg B5 (Gamborg *et al.* 1968) or Murashige and Skoog (1962), is still a common approach when establishing a new cell culture. A more recently reported, fast and pragmatic approach is to optimise the growing medium of the *in vitro* culture by analysing the mineral proportions of the intact plant material (Gonçalves *et al.* 2005). In order to understand the complexity of the requirements of a novel culture, as well as to reduce the number of experiments and the time required, a more comprehensive approach is needed. Systematic optimisation is nowadays frequently used with microbial cultures and, in recent years, it has also been applied to plant cell cultures. The most widely used design has been the central composite design (Eilers *et al.* 1988, Tuominen *et al.* 1989, Nuutila *et al.* 1991, Toivonen *et al.* 1991, Chattopadhyay *et al.* 2003) developed by Cochran and Cox (1957) which employs regression analysis. Response surface methodology is then applied to map the expected performance of a range of response variables in a multi-variable system. In these studies, interactions between variables were found to markedly affect the system. Methods designed for slowly growing cultures that are based on several initial experiments as the starting points have also

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been published (Tammisola *et al.* 1993). These methods enable a more simultaneous approach, thus making it possible to avoid time-dependent variables such as seasonal or even genetic changes.

Nyiredy *et al*. (1988) developed the PRISMA model, *i.e*., a prismatic solvent mixture design for analytical purposes. Combination of the desirability functions introduced by Deming (1991) with the PRISMA design has been successfully used in HPLC optimisation (Outinen *et al.* 1998). In the present study, the PRISMA model was used for systematic optimisation of plant culture media. A method called CELLOP, which combines medium mixture design and statistical evaluation and modelling of the response variables by means of desirability functions for predicting the behaviour of the response variables, was constructed.

The major nutrients calcium, inorganic nitrogen, and sucrose were selected as the explanatory mixture variables due to their marked effects on growth, biosynthetic activity and somatic embryogenesis in several species of *Apiaceae* family such as *Peucedanum palustre* (Vuorela *et al.* 1993), *Angelica archangelica* (Eeva *et al.* 2003), and *Daucus carota* (Overvoorde and Grimes 1994).

Materials and methods

Plants: Embryogenic cell lines of angelica *Angelica archangelica* L. subsp. *archangelica, Apiaceae* (Eeva *et al.* 2003) grown on a 75 % (v/v) hormone-free Gamborg B5 basal medium (Gamborg *et al.* 1968), and hogs fennel *Peucedanum palustre* (L.) Moench, *Apiaceae* (Vuorela *et al.* 1993) grown on a hormone-free Gamborg B5 basal medium, were used as model cultures (see Table 1 and Fig. 1). The size of the inoculum was 1.00 g fresh mass per flask. The cultivations were carried out at 25 ± 2 ºC on a rotary shaker at 90 rpm using a 16-h photo-period (irradiance of 30 μmol m^{-2} s^{-1}). In the growing experiments the material was sub-cultured three times in 100-cm^3 conical flasks with 20.0 cm^3 of fresh medium in order to minimise the carry-over effect caused by the original medium. After the 3rd sub-culturing, the plant material was visually examined for embryogenic activity.

The experiments were made simultaneously with both cultures and 20 different experimental culturing medium mixtures (Table 1, Fig. 1*B*) designed with PRISMA culturing medium mixture design with 8 replicates in each medium. Three nutrients, calcium (x_1), inorganic nitrogen (NO_3^-/NH_4^+) (x_2), and sucrose (x_3) were the explanatory variables in the design. The inorganic salts were added to the media as stock solutions prior to pH adjustment, and then autoclaved.

Coumarin analysis: After the growing experiments, the plant material was removed from the medium by vacuum filtration and lyophilised. A 100-mg sample of dried plant material was extracted with 3.00 cm^3 of methanol (*Rathburn Chemicals Ltd*, Walkerburn**,** UK, HPLC-grade) in a sonication bath for 30 min. After extraction,

Table 1. Molar concentrations [mM] of the explanatory variables Ca^{2+}, NO_3^-/NH_4^+, and sucrose in the used medium mixtures designed with the PRISMA. The total strength of the media used in the *A. archangelica* is 75 % (v/v) of those used in the *P. palustre* experiments.

Experimental data points in PRISMA design			*A. archangelica*			*P. palustre*		
			[Ca^{2+}]	[NO_3^- / NH_4^+]	[Sucrose]	[Ca^{2+}]	[NO_3^- / NH_4^+]	[Sucrose]
0.1	0.1	0.8	0.25	6.25 / 0.50	105.0	0.33	8.33 / 0.67	140.0
0.1	0.3	0.6	0.25	18.70 / 1.50	78.9	0.33	25.00 / 2.00	105.0
0.1	0.6	0.3	0.25	37.50 / 3.00	39.4	0.33	50.00 / 4.01	52.6
0.1	0.8	0.1	0.25	50.00 / 4.00	13.1	0.33	66.70 / 5.34	17.5
0.3	0.1	0.6	0.75	6.25 / 0.50	78.9	1.00	8.33 / 0.67	105.0
0.3	0.3	0.3	0.75	18.70 / 1.50	39.4	1.00	25.00 / 2.00	52.6
0.3	0.3	0.4	0.75	18.70 / 1.50	52.6	1.00	25.00 / 2.00	70.1
0.3	0.4	0.3	0.75	25.00 / 2.00	39.4	1.00	33.30 / 2.67	52.6
0.3	0.6	0.1	0.75	37.50 / 3.00	13.1	1.00	50.00 / 4.01	17.5
0.4	0.1	0.5	1.00	6.25 / 0.50	65.7	1.33	8.33 / 0.67	87.6
0.4	0.3	0.3	1.00	18.70 / 1.50	39.4	1.33	25.00 / 2.00	52.6
0.4	0.5	0.1	1.00	31.20 / 2.50	13.1	1.33	41.70 / 3.34	17.5
0.5	0.1	0.4	1.25	6.25 / 0.50	52.6	1.67	8.33 / 0.67	70.1
0.5	0.2	0.3	1.25	12.50 / 1.00	39.4	1.67	16.70 / 1.34	52.6
0.5	0.3	0.2	1.25	18.70 / 1.50	26.3	1.67	25.00 / 2.00	105.0
0.5	0.4	0.1	1.25	25.00 / 2.00	13.1	1.67	33.30 / 2.67	17.5
0.6	0.1	0.3	1.50	6.25 / 0.50	39.4	2.00	8.33 / 0.67	52.6
0.6	0.2	0.2	1.50	12.50 / 1.00	26.3	2.00	16.70 / 1.34	35.1
0.6	0.3	0.1	1.50	18.70 / 1.50	13.1	2.00	25.00 / 2.00	17.5
0.8	0.1	0.1	2.00	6.25 / 0.50	13.1	2.67	8.33 / 0.67	17.5

the samples were centrifuged (1500 *g*) for 15 min. The supernatant was used as sample for HPLC analysis.

The coumarin content was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) according to the method described in Eeva *et al.* (2003). Injected sample volumes were 0.050 cm^3 (*A. archangelica* samples), and 0.030 cm^3 (*P. palustre* samples). Bergapten (*Fluka Chemie*, Buchs, Switzerland) was used as an external standard for *A. archangelica*, and umbelliferone (*Sigma*, St. Louis, USA) for *P. palustre*.

Mathematical and statistical methods: As is Outinen *et al.* (1998), the data was processed by *MATLAB version 5*, student edition (*Mathworks*, Sherbon, MA, USA) using the data analysis toolbox on *MATLAB* (*Profmath*, Helsinki, Finland). A windowed graphical user interface was created for data handling and graphical assessment of the response surfaces.

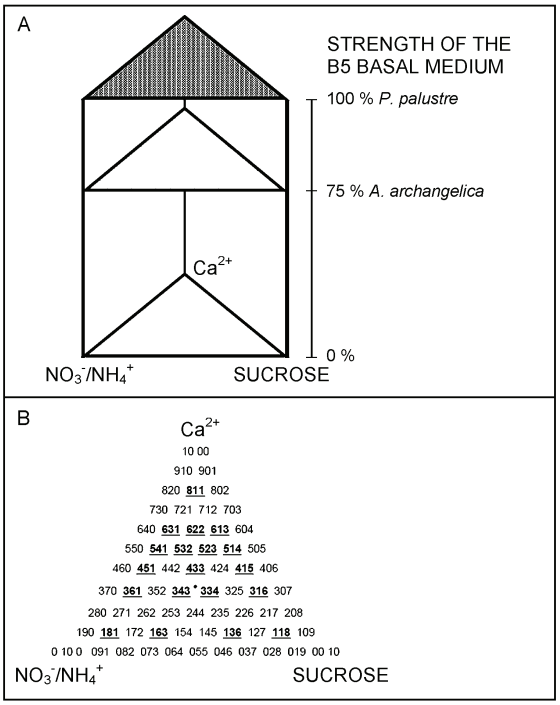

Fig. 1. The three-dimensional medium mixture PRISMA design in the CELLOP model (*A*) showing the horizontal part symbolising the ratios of the selected explanatory variables and the vertical part symbolising the total strength of the media. The medium mixture points describing the horizontal plane in the CELLOP model (*B*). Each 3-number data point symbolises a different possible combination of the explanatory variables, the sum of the mixture component levels equalling 10. The experimental data points examined are underlined. The middle point (3.33:3.33:3.33) is illustrated by (*closed circle*). For details see Materials and methods.

Mixture design: The CELLOP model can be visualised as a three-dimensional geometric design, a prism (Fig. 1*A*), in which the data points (P_d) in the horizontal part of the design represent different culturing medium mixtures (Fig. 1*B*). Thus the data points in the design symbolise quaternary (3 explanatory variables), ternary (2 explanatory variables) or binary (1 explanatory variable) variable mixtures when water is taken into account as the fourth variable. The data points can be depicted as three-number coordinates that represent concentration ratios of the selected explanatory variables calcium, inorganic nitrogen *i.e.* NO_3^-/NH_4^+, and sucrose, calculated so that the middle point 0.333:0.333:0.333 is the hormone-free Gamborg B5 basal medium (Table 1). Unlike in the mobile phase optimisation of high-performance liquid chromatography (Outinen *et al.* 1998), all the selected explanatory mixture variables are essential to the plant cells. Therefore only quaternary mixtures were used that contained at least the minimum amounts of the explanatory nutritional variables. The vertical dimension symbolises the total strength of the medium mixtures. For the *A. archangelica* cultures the culturing media were diluted to 75 % of the original strength (Fig. 1*A*) because, in preliminary experiments, the growth of the *A. archangelica* culture deteriorated in the full strength Gamborg B5 basal medium.

Regression models: The aim of the statistical modelling in this study was to construct models that predict the behaviour of the plant material in unexamined areas of the experimental set-up on the basis of the data obtained from the growing experiments in order to find optimal conditions for selected products, *i.e.*, growth and coumarin production. As the sum of the mixture component levels always equal one, the models for the responses (R_p) can be written in terms of two of the nutrients only. The regression models for the responses were used to define the optimum in CELLOP. Possible types for the regression models are a linear (Eqn. 1), a full quadratic (Eqn. 2), or a canonical model in which the data points $y_1 ... y_y$ are described explicitly.

$$y = b_0 + b_1 x_1 + b_2 x_2 \quad (1)$$

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{1,1} x_1^2 + b_{1,2} x_1 x_2 + b_{2,2} x_2^2 \quad (2)$$

The best fitting regression model can be selected for each response variable separately. All combinations of the given regression models are possible in the developed system. This is often required in order to better describe the characteristics of the biological material being examined.

Desirability functions: Comparison of various response variables in a multivariable system is often complicated due to the different dimensions of the responses and their units, and thus can not be processed using simple mathematical functions (Deming 1991). In the optimisation of a production process economical aspects must also be considered. Attempts to reach the overall optimum often require a combination of conflicting aims. The nutritional requirements of the culture are usually highly dependent on the aim, *e.g.* a high sucrose concentration in the medium enhances biomass production to a certain point, but deteriorates biosynthetic activity and/or stimulates embryo formation.

Optimisation of the growing conditions therefore requires weighing of the individual factors affecting the system and compromises are inevitable. The desirability functions provide a means for defining the "desirable" and "undesirable" values of separate responses in the investigated system, and for combining them in an overall desirability function. The desirabilities for each response variable are defined separately, the value 0 being given for a completely unsatisfactory lever of response and 1 when the desired level has been reached.

The desirability functions of individual response variables convert the calculated response values of dry mass (DM) (y_1) [g dm^{-3}(medium)], and coumarin concentration (y_2) [μg g^{-1}(DM)] into the unitless desirability values (D): $0 \le D\ (R_p) \le 1$. In this study, the desirability for the response, $D(R_p)$, is expressed using a logistic function $D(R_p) = 1/1 + e^{(-Rp-Rp0)/\delta}$, where R_p0 is the selected "mean response value" and δ is the "deviation" selected on the basis of the expected characteristics of the system. The overall desirability (D_o) is defined as the product, geometric mean $D_o = (d_1\ d_2....d_m)^{(1/m)}$ where $d_1\ d_2....d_m$ are the desirability functions of each response variable.

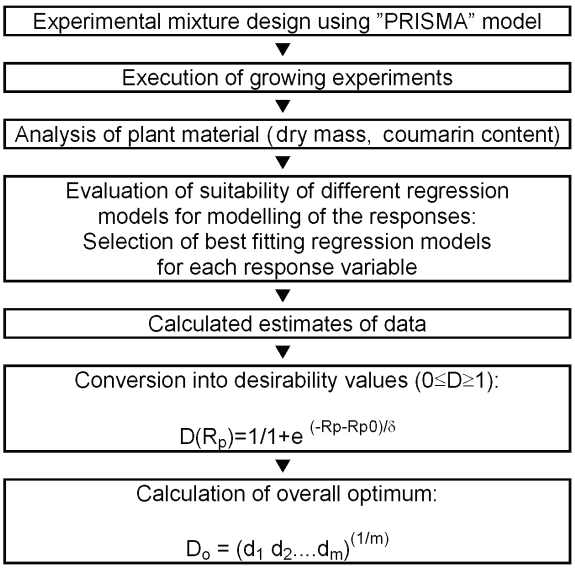

Fig. 2. Flow chart describing the separate steps in the optimisation process in CELLOP.

Results and discussion

The average dry mass (DM) after the third sub-culturing phase of the *A. archangelica* cultures (n = 160) was 9.55 ± 2.80 g dm^{-3}(medium). According to this, the "mean response value" R_p0 was set to 9.55 and "deviation" δ = 2.80, R_p0 = 9.55; ($6.75 \le R_p \le 12.4$) (Fig. 3*A*). The average coumarin content of the *A. archangelica* cultures was 5160 ± 2270 μg g^{-1}(DM). Thus R_p0 = 5160; ($2890 \le R_p \le 7430$) (Fig. 3*B*). The respective values for *P. palustre* were DM: R_p0 = 10.75; ($5.85 \le R_p \le 15.65$) and coumarin content: R_p0 = 14790; ($8330 \le R_p \le 21250$).

The developed system enabled the use of both linear and full quadratic regression models. The r^2 values of the model *vs*. test analysis revealed that the full quadratic model predicted the behaviour of the DM and coumarin content in both model cultures better than the linear model. The model *vs*. test with a full quadratic regression gave r^2 = 0.8201 for the DM and r^2 = 0.6961 for the coumarin content, whereas the linear regression led to r^2 = 0.7380 for DM and r^2 = 0.6550 for the coumarin content. Thus the full quadratic model was used in calculating the final results. It also became clear that the biomass production was easier to predict than the coumarin content because the r^2 values were clearly higher for the DM despite the regression model used.

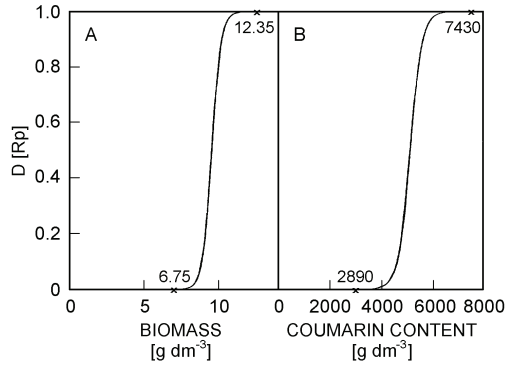

Fig. 3. The logistic desirability functions used for the DM of *A. archangelica* cultures (*A*) and the coumarin content of *A. archangelica* cultures (*B*). $D(R_p)$ = desirability of the response.

The theoretical overall optima for the best conditions were easily read from the contour plot inside the horizontal plane of the CELLOP. The model suggested one clear overall optimum area for both cultures in the experiments (Figs. 4*C*,*F*), which could be repeated.

With *A. archangelica* the overall optimum was located near the sucrose corner 0.1:0.1:0.8 (Fig. 4*C*), in which the highest dry mass and coumarin content were reached, dry mass being 24.6 % and the coumarin content 40.5 % higher than in the standard Gamborg B5 medium (0.33:0.33:0.33) (Fig. 4*A*,*B*). The calculated overall optimum point with regard to the calcium, inorganic nitrogen, and sucrose concentrations of the medium was 0.19:0.08:0.77, *i.e.* 0.47 mM Ca^{2+}, 5.06 mM NO_3^-, 0.40 mM NH_4^+, and 96.25 mM sucrose, giving a total desirability of D_o = 1.0. This is in agreement with preliminary observations (Eeva *et al.* 2003) in which, out of the examined sucrose concentrations, the highest DM and coumarin content were reached in medium containing 3 %, *i.e.*, 87.7 mM sucrose.

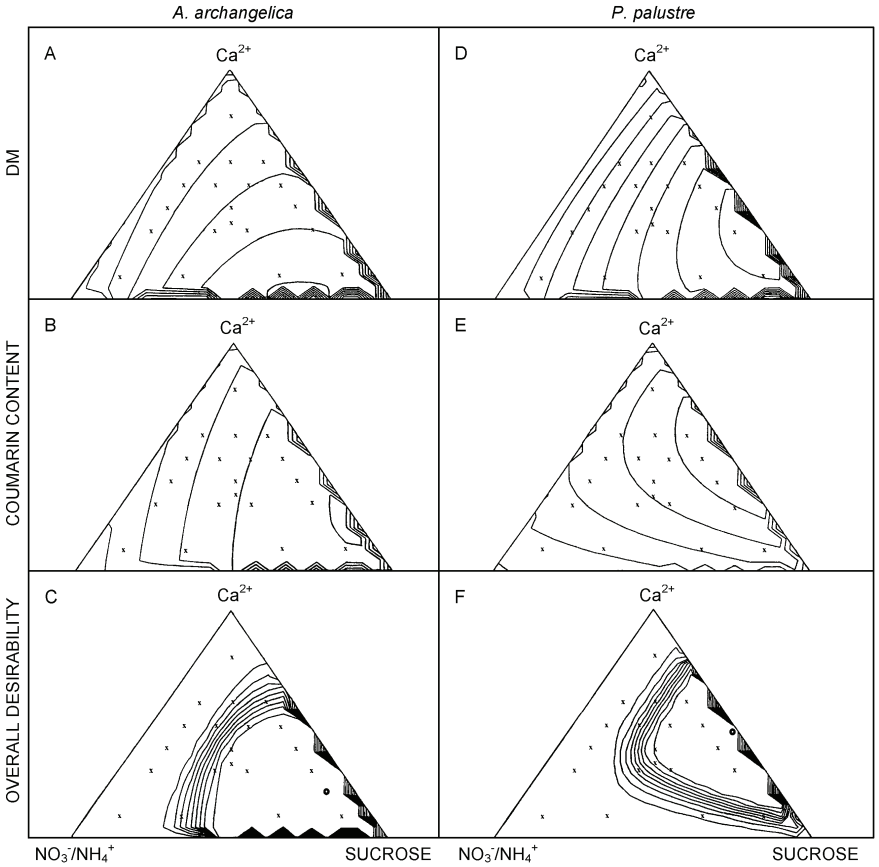

Fig. 4. Desirability surfaces of the DM (*A*), coumarin content (*B*) and overall desirability (D_o) (*C*) for *A. archangelica* cultures. Desirability surfaces of the DM (*D*) and coumarin content (*E*) and overall desirability (D_o) (*F*) for *P. palustre* cultures are calculated on the basis of the data from all 20 experimental data points. The examined data points (medium mixtures) in Fig. 4 are depicted by *crosses*. The most favourable mixtures in *C* and *F* are depicted by (*open circles*).

For *P. palustre* the overall optimum area occurred between the calcium (0.8:0.1:0.1) and sucrose (0.1:0.1:0.8) corners (Fig. 4*F*). When the maxima of the different responses in the model were compared, the DM maximum was close to the sucrose corner (Fig. 4*D*), while the coumarin content was highest between calcium and sucrose corners (Fig. 4*E*). The highest dry mass was reached in point 0.3:0.1:0.6, and was 61.8 % higher than in the standard Gamborg B5 medium. The coumarin content was highest in point 0.5:0.1:0.4 and exceeded the reference by 58.1 %. The amount of nitrogen needed for growth and coumarin production was found to be markedly low. Thus the calculated optimum with regard to the same three variables was 0.48:0.03:0.49, *i.e.*, 1.60 mM Ca^{2+}, 2.84 mM NO_3^-, 0.23 mM NH_4^+, and 85.10 mM sucrose, giving a total desirability of $D_o = 1.0$.

Visual evaluation of the plant material revealed that the variation in the concentrations of the selected explanatory variables clearly affected the embryo formation and differentiation (shoot/root ratio). The 1.25 mM Ca^{2+} increased embryo formation of the *A. archangelica* cultures the most (Fig. 5*A*). Reports on other embryogenic cell lines such as carrot (Overvoorde and Grimes 1994) and *Hevea brasiliensis* (Etienne *et al.* 1997) support this finding as well. In the *P. palustre* experiment, the inorganic nitrogen concentration was the key factor for embryo formation (Fig. 5*B*). The same has also been reported for *Betula pendula* cultures (Nuutila *et al*. 1991). However, it should be emphasised that sugar played an important role in embryo formation with both of the cultures studied. This is in full agreement with the results of Jeannin *et al.* (1995) and Eeva *et al.* (2003). The developed model enables to weigh the required or desirable features of the examined material. This could for instance be the morphology, *i.e*. the shoot/root ratio, embryogenic activity or biosynthetic activity. The explanatory variables used in this study make it possible to use the experimental set-up, *e.g*. in the optimisation of *in vitro* propagation procedures or in the development of two-stage culturing methods for bioprocesses (Eeva *et al.* 2003, Chen *et al.* 2004).

The variation in biomass production in the *A. archangelica* experiments was smaller than that in the *P. palustre* experiments, the relative standard deviation in the average biomass of *A. archangelica* cultures ($n = 160$) being RSD = 29.4 % and of *P. palustre* cultures RSD =

45.6 % (n = 160). The smaller changes in the absolute concentrations of the nutrients in the *A. archangelica* media could explain this finding. This might also have caused smaller variation between the replicates in the same medium. The total medium strength had no effect on the variation in the overall average coumarin content of either cultures (relative standard deviations for *A. archangelica* RSD = 44.0 % and for *P. palustre* RSD = 43.7 %, respectively, n = 160).

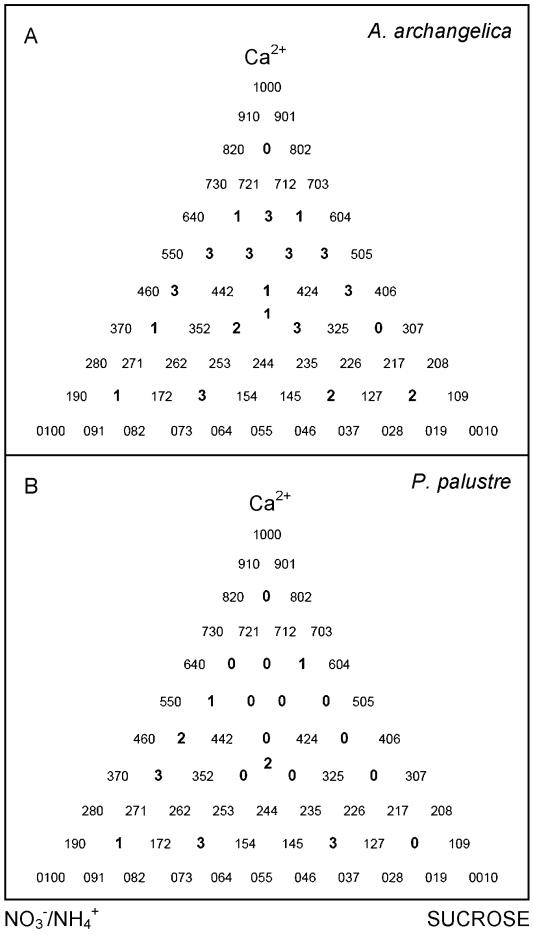

Fig. 5. Embryo formation in the medium mixture points in cultivation experiments of *A. archangelica* (*A*) and *P. palustre* (*B*). 0 = no embryos, 1 = low number of embryos, 2 = moderate number of embryos, 3 = high number of embryos. Sum of the mixture component levels equals 10.

The total duration of the optimisation experiment is critical for slowly growing cultures, especially with the classical approach involving several sequential experiments. Methods suitable for slowly growing cultures have been developed, *e.g.*, by Tammisola *et al.* (1993), but the number of experimental steps may still easily increase. For instance, the duration of a 5-step study with 3 sub-culturing phases is over 10 months. A differentiated culture, especially the *A. archangelica* line, is markedly affected by seasonal changes (Eeva *et al.* 2003). Thus a series of experiments lasting longer than 10 months is easily affected by this phenomenon, and a faster approach such as CELLOP is a necessity.

During data processing it was noted that the reduction of the experimental data points from 20 to 13 by excluding data points 0.4:0.1:0.5, 0.4:0.5:0.1, 0.5:0.1:0.4, 0.5:0.2:0.3, 0.5:0.3:0.2, 0.5:0.4:0.1, 0.6:0.2:0.2 did not change the overall optimum or the desirability of either cultures. However, it was not possible to further reduce the number of data points. The number of replicate flasks could also be randomly reduced from 8 to 3 without any marked change in the overall optimum or desirability.

The developed model enables systematic optimisation of the growth medium for plant cell cultures within a relatively short period of time. It also enables the optimisation of two-phase cultivation systems with only one growing experiment. In this work, although the plants from which the cultures were derived are closely related, they still showed significant differences both in growth and coumarin production as well as in embryo formation, thus making individual optimisation for each culture necessary. The developed model also enables the determination of absolute concentrations of the chosen explanatory variables for new cultures by using the vertical dimension of the prism.

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The second volume of book series "Genetic Resources, Chromosome Engineering, and Crop Improvement" loosely continues with previous volume devoted to grain legumes. This volume consists of 13 chapters dealing with major cereal crops such as wheat, rice, maize, oat, barley, pearl millet, sorghum, rye and triticale. Each chapter provides a general comprehensive account of the crop, its origin, wild relatives, exploitation of genetic resources in gene pools through breeding and cytogenetic manipulation, and genetic enrichment using the tools of molecular genetics and biotechnology. The main accent is put to present stage of breeding technologies accompanied by advanced techniques of cytogenetics and physical mapping. Valuable compilations about, *e.g.*, genetic enhancement or cytogenetic manipulations in respect to each of cereals are given to map the current knowledge in the field. A broad overview is provided for readers about cytogenetic architecture of cereals, breeding strategies and techniques like polyploidization and hybridization. Molecular biology techniques (*e.g.*, RFLP, comparative mapping) are represented in connection with physical mapping of chromosomes or cytogenetic manipulation. Since the book is intended for scientists, professionals, and students interested in improvement of crops in general and cereals in particular, the ethical issues are not discussed. Text is supplemented by 15 pages of colour figures which nicely illustrate an impact of cytogenetic and breeding results.

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