Unbiased estimation of the proportion of non-embryogenic cell clusters in the somatic embryogenic culture of Douglas-fir

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Introduction

Somatic embryogenesis is the process in which embryos, similar to their zygotic counterparts, are induced to develop in culture from somatic cells. It is a powerful tool for clonal *in vitro* propagation of selected trees, including conifer species, and a suitable model system for investigation of embryo development regulation. Embryogenic cultures of Douglas-fir were induced from immature zygotic embryos (Fig. 1A, B, C). They proliferate as embryonal masses (EMs; Fig. 1D) consisting of polyembryogenic centres (Fig. 1E, 3A,B) of various sizes and singulated somatic embryos (SEs; Fig. 3B,C). These embryonal structures are composed of two types of cells – meristematic cells with prominent nuclei and dense cytoplasm and vacuolated and elongated suspensor cells. However, in some lines of Douglas-fir proliferating EMs, non-embryogenic cell (NECs) clusters interspersed with early SEs were observed (Gautier et al 2017) (Fig. 2A, B; 3C,D).

In order to evaluate the differences between lines we wanted to quantify the proportion of the SEs and NECs in the embryonal mass. For estimation of this proportion (volume density) we used stereological point-counting method based on counting points of the test grid falling in the tissue under study. Stereological evaluation based on systematic uniform random sampling yielded unbiased estimation of parameters under study.

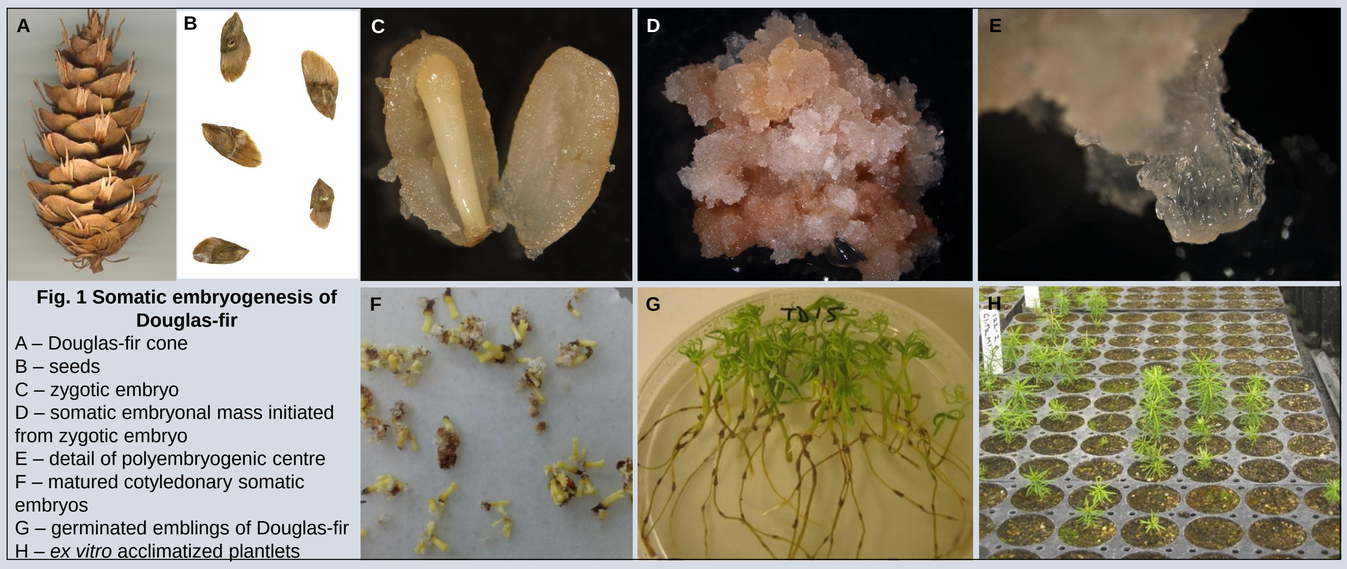
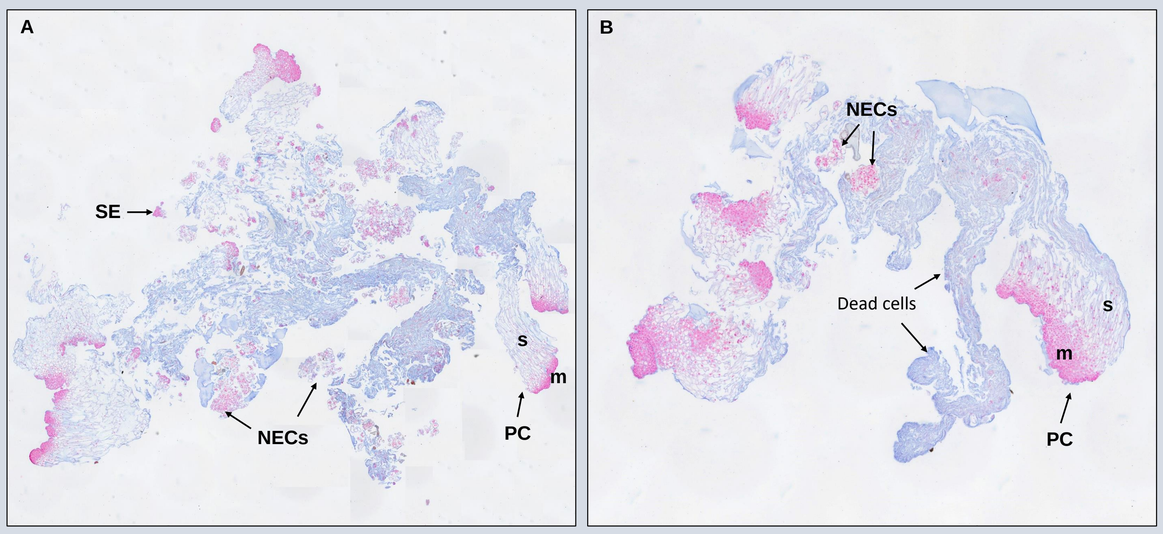


Fig. 1 Somatic embryogenesis of Douglas-fir
A – Douglas-fir cone
B – seeds
C – zygotic embryo
D – somatic embryonal mass initiated from zygotic embryo
E – detail of polyembryogenic centre
F – matured cotyledonary somatic embryos
G – germinated emblings of Douglas-fir
H – *ex vitro* acclimatized plantlets

Fig. 2 Representative images of EM sections

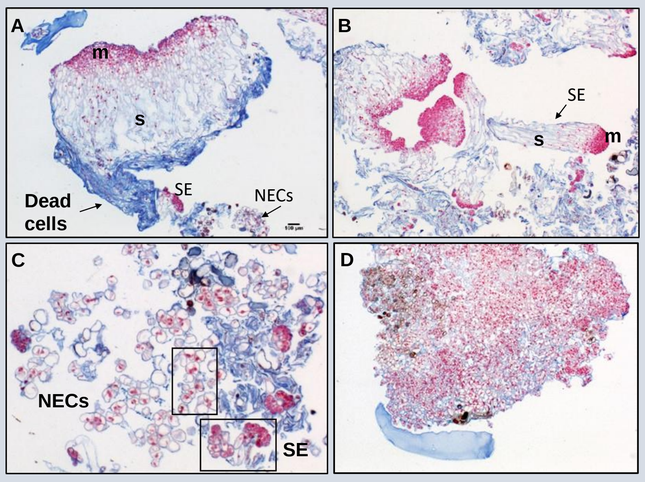


Embryonal masses of both lines consists of polyembryogenic centres (PC) and singulated embryos (SE) composed of meristematic cells (m; prominent nuclei are stained in red) and suspensor cells (s; cell walls are stained in blue). Blue are also remnants of dead cells. Except of SEs, NEC clusters are present in EMs of both lines. A – line TD17, B – line TD17-1

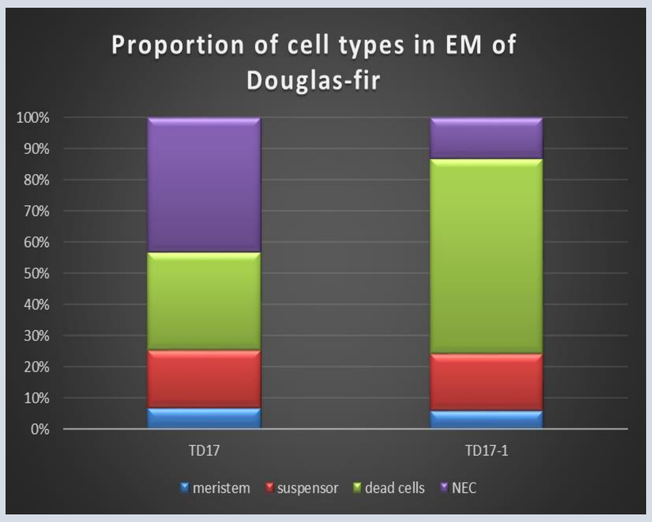
Material and Methods

- Douglas-fir somatic embryonal mass of two lines (TD17 and TD17-1) was cultivated *in vitro.*
- 5 random samples from each line were fixed, dehydrated, infiltrated with paraffin, cut (section thickness 12 µm) and stained with Alcian Blue and Nuclear Fast Red in order to visualized cell walls (in blue) and cell nuclei (in red).
- Stereological point-counting method was applied via stereological plug-in module PointGrid based on the software Ellipse (ViDiTo, Košice, Slovakia), enabling to quantify simultaneously several tissue or cell types. The regular grid of points was superimposed on the microscopic images of EMs sections being positioned uniformly at random. Ten slides from each sample were chosen and 1 section from each slide was evaluated. These sections were larger then field of vision, therefore 2-10 images were captured for one section.
- Four cell categories were evaluated – meristematic cells of somatic embryos or polyembryogenic centres, suspensor cells, non-embryogenic cells and dead material that consists of embryo or suspensor cell remnants. Two point grids were superimposed on each image since one grid enable to evaluate only 4 categories, one of them is background. Each cell category was matched with one point colour (Fig. 4).
- Points which hit particular cell categories as well as all points hitting EM sections were counted.
- Proportion of cell categories on the embryonal mass sections area was calculated according to Weibel (1979): estA = P * a; estimated area of cell category is equivalent to the product of number of particular point hits (P) and the area of one testing point of the grid (a).

Fig. 3 Examples of evaluated structures



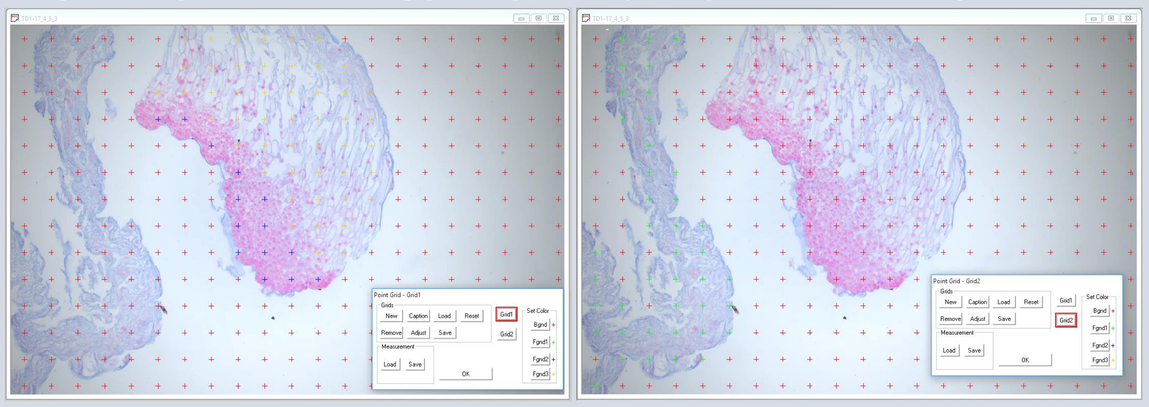
A – large polyembryogenic centre (PC) with broad meristem (m) and huge suspensor (s) ending with „anchor“ of dead suspensor cells; in the vicinity of this part small SE and cluster of NECs are localized
B – PCs and large singulated embryos (SE) with distinct meristematic head (m) and long suspensor (s)
C – clusters of loosely arranged non-embryogenic cells (NECs) in the proximity of small SEs
D – large compact cluster of NECs



Results

Three samples from each line (i.e. 162 and 169 images for TD17, resp. TD17-1) have been evaluated till now. From these preliminary data it is quite clear that line TD17 produces more non-embryogenic cells then TD17-1 (43% / 13%). Surprisingly, high proportion of TD17-1 EM is composed of dead cells (63% / 31%). It could be caused by presence of huge polyembryogenic centres that are connected with other structures of EM by „anchor“ of dead cells that are localized in the end of suspensor. This part can be very large. Meristematic and suspensor cells represent similar proportions in both lines (6%, resp. 18%).

Fig. 4 Sample of the testing point grids superimposed on the image



Conclusions

The stereological point-counting method using a regular grid of points (which is positioned uniformly at random on the section) proved to be a very effective tool for the estimation of the volume density (proportion) of non-embryogenic cell clusters within embryonal mass of Douglas-fir. Even though only a part of samples were evaluated, results confirmed our presumption based on the microscopic observations. However, we need to finish the evaluation of remaining samples in order to get a large enough data set. Application of another test grid with higher density of testing points could be useful.

Acknowledgements:
This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (grants no. LTC 17030 and NPUI LO1417)

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