A statistical comparison of various factors on embryogenic proliferation, morphogenesis and regeneration in *Lolium temulentum* cell suspension colonies

S.J. Dalton & I.D. Thomas

*AFRC Institute of Grassland and Environmental Research, Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, UK*

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**Abstract**

Cell suspension colonies from four embryogenic *Lolium temulentum* lines were selected and plated individually in 25 embryoid maturation treatments which varied in various factors reported to stimulate embryogenesis or improve regeneration. Using a numerical scoring system to compare the cultures against a control, treatments were identified which increased growth, suppressed morphogenesis or encouraged premature shoot formation.

No treatment significantly improved the proportion of colonies with globular or mature embryoids, but some prevented maturation and increased the proportion with translucent embryogenic proliferation. Other treatments accelerated maturation causing increased de-differentiation of embryogenic tissues. These treatments also tended to discourage the differentiation of discreet embryoids.

Colonies were later transferred *en masse* to a regeneration medium and scored using another numerical system. Embryoid maturation conditions were then identified which increased or suppressed subsequent shoot regeneration. The two scoring systems enabled cultures of the four lines to be characterised in detail and identified somatic variation in embryogenic development, morphogenesis and de-differentiation.

**Introduction**

The importance of embryogenesis as a pathway to shoot regeneration has been acknowledged in many studies (Thorpe 1988). However, the presence of embryogenic proliferation or of embryoids is generally assessed in a destructive way by sectioning (Ho & Vasil 1983a) or in a subjective way by appearance (Heyser et al. 1983). The stages of embryogenic development are relatively easily recognised by shape in some species such as carrot (Thorpe 1988), but gramineous embryos tend to increase in size, complexity, hardness and opacity rather than change shape, while kernel maturity has traditionally been assessed on the qualities of the endosperm rather than the embryo. Although it is acknowledged that species differ considerably in the ease of recognising embryogenic proliferation, somatic embryogenic proliferation has been described in wheat (He et al. 1986), rice (Heyser et al. 1983), maize (Lu et al. 1983), sugar cane (Ho & Vasil 1983b) and in a range of cereals (Nabors et al. 1983).

The reporting of the percentage of cultures which give rise to embryogenic proliferation or plants is common, but only rarely have statistically proven effects of treatments on the num-
bers of embryoids produced been shown (Gray & Conger 1985). This work used liquid cultures in which individual embryoids could be identified. Otherwise, the difficulty of counting embryoids and the subjective nature of scoring, cause much published research to indicate only that mature embryoids, or sometimes, only that compact tissues were present. In addition, time of scoring affects the percentage of cultures with embryogenic tissues recorded, if only those with mature stages are counted, when less mature and even de-differentiating tissues which were previously 'embryogenic' may be present.

More embryoids or embryogenic tissue is generally considered better because regeneration is increased, with success usually being defined as increasing numbers of shoots. However, for many experiments, this does not indicate efficient regeneration, but only secondary proliferation of some embryoids (Bhaskaran et al. 1988). In an ideal culture, pro-embryoids which were extant at the end of a primary proliferative culture phase would mature into somatic embryoids during a secondary phase and would regenerate via germination during a tertiary phase. Further proliferation during the secondary phase may be a source of genetic variation and cause a range of development stages to be present.

Scoring criteria have been compiled to compare and analyse subjective characters at a whole plant level (Seidewitz 1974) and at a sward level (Hodgeson 1976). Similar scoring methods covering embryogenic development and morphogenesis, should therefore enable cultures to be analysed in a similar way to field experiments.

The efficacy of this approach was tested on cell suspension colonies of *Lolium temulentum* by comparing various factors reported to promote embryogenesis or regeneration. Embryogenic proliferation and the formation of pro-embryoids in primary phase cultures have been improved by darkness (He et al. 1986), the addition of abscisic acid (Ho & Vasil 1983), tryptophane (Sirirawdana & Nabors 1983; Nabors et al. 1983), proline (Green et al. 1983; Strickland et al. 1987), dicamba (Hanning & Conger 1982), casein hydrolysate (Abe & Futsuhara 1986), and both in combination (Gray & Conger 1985), as well as high sucrose levels (Lu et al. 1983). Maturation of pro-embryoids in secondary phase culture has been improved by darkness (He et al. 1986), the addition of dicamba (Zimny & Lorz 1986), casein hydrolysate (Abe & Futsuhara 1986), and abscissic acid (Kott & Kasha 1984), and by using maltose, malt extract and fructose-based media (Strickland et al. 1987). Finally, in tertiary phase regeneration culture the frequency of shoot production from embryogenic tissue has been improved by optimising the callus to medium density and conditioning the medium (Raghava Ram & Nabors 1985), the use of maltose-based medium (Strickland et al. 1987) and the addition of tryptophane (Raina et al. 1987). Some additives have improved all three culture phases: silver nitrate (Purnhauser et al. 1987) and cefotaxime (Mathias & Boyd 1986).

Other culture factors considered potentially important through experience were washing colonies with 2,4-D free medium, the solidifying agent, the method of sealing dishes and the freshness of the medium.

**Materials and methods**

**Culture material**

The cultures used were four embryogenic cell suspension lines derived from embryogenic shoot tip cultures from four seedlings of *Lolium temulentum* accession BA 3081. Chopped embryogenic calli were placed in 10 ml suspension induction medium in 180 ml bottles and shaken at 100rpm under diffuse light (25 μmol m⁻² sec⁻¹) at 25°C. After four weeks, proliferating cultures were transferred to 250 ml flasks containing 30 ml suspension maintenance medium and after increasing the volume for a further two weeks, the cultures were maintained by a weekly 1 to 4 subculture regime (15 ml suspension colonies in medium added to 60 ml fresh medium).

Four cell suspension lines were selected and each was replicated into four flasks and maintained for three subcultures before the experiment. The cell suspension colonies were plated 29 to 31 weeks after suspension induction.

Seven to nine day old cell suspension colonies (1–2 mm diam) were plated onto 10 ml embryo maturation medium in 60 mm petri dishes using a