Review of methodologies and a protocol for the Agrobacterium-mediated transformation of wheat
Jones, Huw D; Doherty, Angela; Wu, Huixia

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Abstract
Since the first report of wheat transformation by *Agrobacterium tumefaciens* in 1997, various factors that influence T-DNA delivery and regeneration in tissue culture have been further investigated and modified. This paper reviews the current methodology literature describing *Agrobacterium* transformation of wheat and provides a complete protocol that we have developed and used to produce over one hundred transgenic lines in both spring and winter wheat varieties.

Introduction
Transformation of cereal crops is a powerful research tool for gene discovery and function to investigate genetically controlled traits and is fast becoming a key element in the process of varietal improvement. It provides key underpinning knowledge to inform and short-cut conventional breeding strategies. For specific crops, it also enables the introduction of novel genes directly into locally-adapted germplasm and the creation of new genetically modified varieties. As testament to this, a total of 81 million Ha of approved GM crops, mainly for herbicide tolerance or insect resistance, were planted in 2004 [1], although wheat does not currently form part of this portfolio.

Wheat was among the last of the major crops to be transformed with the first fertile transgenic plants being reported using particle bombardment little over a decade ago [2-6]. Advances in the design of micro-projectile devices, choice of explant, media composition and selection systems has enabled the application of wheat transformation to study the role specific genes in a wide range of agronomically important traits (reviewed by [7-9]). Particle bombardment remains a robust, relatively efficient method for the genetic manipulation of wheat [10], however at the molecular level, the DNA integration sites are often unnecessarily complex. There are several significant advantages to transferring DNA via *Agrobacterium*, including a reduction in transgene copy number, the stable integration with fewer rearrangements of long molecules of DNA with defined ends and the ability to generate lines free from selectable marker genes [7,11-14]. This has been a driving force in the development of methods using *Agrobacterium tumefaciens* to deliver DNA although the ability to routinely transform wheat in this way is currently restricted to a few, well-resourced public and commercial laboratories worldwide. This is partly due to the need for experienced personnel and expensive laboratory and plant growth infrastructure but also through a lack of clearly-written, complete, publicly-available protocols. There are several research papers and patents describing specific improvements to methodologies but these fail to provide a step-by-step guide to the transformation process as a whole.

We have compared the published literature under headings that describe the main variables in the
transformation process. First, we consider the relatively narrow range of wheat genotypes that have been successfully transformed, the choice of explant and the pre-treatments that were carried out. Second, we compare the Agrobacterium strains, resident Ti plasmids and binary vectors used and consider the importance of additional virulence genes. The various inoculation and co-cultivation conditions are discussed and finally the key steps to control the overgrowth of Agrobacterium cells and the selection of regenerating transformed plants are described. We then provide a detailed protocol for the transformation of freshly isolated immature embryos and regeneration of fertile plants in 9–12 weeks.

**Genotype and explant pre-treatments**

Immature embryos of Bobwhite, pre-cultured for between 1 and 6 days on CM4C medium, are the most commonly used explant [15-18], although the use of 9 day pre-cultured immature embryos of cv. Fielder [19] and callus derived from immature embryos of Bobwhite [17] and cv. Veery 5 [20] has also been reported (see Table 1 for summary). Although immature embryos of Bobwhite are commonly pre-cultured prior to inoculation, Cheng et al. [17] report no significant difference in transformation efficiencies between immature embryos, pre-cultured ones or embryogenic callus. In an alternative approach, freshly isolated immature embryos of the winter and spring wheat cultivars Florida and Cadenza were found preferable to pre-cultured ones [21] and it is this explant type that is described in the accompanying protocol as it has potential to be applied to other varieties. Precocious zygotic germination is a significant problem when using immature embryo explants but can be suppressed by the addition of hormones such as dicamba, abscisic acid or high levels of 2,4-D to the culture medium. Some authors specifically state that the embryo axis was removed or damaged to prevent zygotic germination [19–21]. A marked effect of embryo size/age on T-DNA delivery and regeneration has been demonstrated, with large embryos (>2 mm) giving significantly higher transient expression levels but lower regeneration frequencies [21] than smaller ones (<1.5 mm). We emphasise the need to use embryos of 0.8–1.5 mm in the accompanying protocol.

### Table 1: Summary of main parameters reported for Agrobacterium-mediated transformation of wheat.

| Wheat variety (S - spring) | Explant type | Embryo Axis removed | Agrobacterium strain (binary vector) | Inoculation (Co-culture) | Control of Agrobacterium cells | Plant selective agent | Transformation Freq. (%) | No of plants reported | Refs |
|---------------------------|--------------|---------------------|--------------------------------------|--------------------------|--------------------------------|-----------------------|------------------------|-----------------------|------|
| Bobwhite (5)              | IE (age NS*) | NS*                 | CSB-ABI (pMON18365)                  | 3 h, 23–25°C             | Carbenicillin (250 mg/l)      | G418                  | 1.4–4.3                | >100                  | [17] |
| Bobwhite (5)              | 4 d PCIE     | NS*                 | CSB-ABI (pMON30139 and others)       | 15–30 min, 23–25°C (2–3 d, 23–25°C) | Carbenicillin (250–500 mg/l) | Glyphosate            | 4.4                    | 3354                  | [16] |
| Bobwhite (5)              | 1–6 d PCIE; 8–30 d EC | NS* | CSB-ABI (pMON18365) | 5–60 min, 23–26°C (2–3 d, 23–26°C) | Carbenicillin (250 mg/l) | G418 | 4.8–19 | 154 | [18] |
| Bobwhite (5)              | 3–6 PCIE     | NS*                 | CSB-1 (pPTN155)                     | 45 min – 3 h, 25°C (1–3 d, 25°C) | Ticarcillin; Vancomycin  | G418                  | 0.5–1.5                | 13                    | [15] |
| Cadenza (S) Florida (W)   | 0–72 h IE    | Yes                 | AGL1 (pALIS4/156)                   | 15 min-5 h, rt* (1–5 d, 24–25°C rt*) | Timentin (160 mg/l) | PPT (L-Phosphinothricin) | 0.3–3.3            | 44                    | [21] |
| Fielder (S)               | 6–9 d PCIE   | Yes                 | AGL0 (pBGX1)                        | 30–60 min rt* (2–3 d, 23–24°C) | Timentin (150 mg/l) | GFP, Bialaphos       | 1.8                 | 4                    | [19] |
| Veery-5 (S)               | 1 d 6-EC     | Yes                 | LBA4404 (pHK21)                     | 15 min at rt* (1 d 27°C, 2 d 22°C) | Timentin (150 mg/l) | Glufosinate ammonium | 1.2–3.9             | 17                    | [20] |
| Vesna (S)                 | IE (age NS*) | NS*                 | LBA4404 (pTOTK233)                  | 15–30 min, (3 d, 27°C)   | Cefotaxime (300 mg/l) | PPT (L-Phosphinothricin) | 0.13–0.41 | 6 | [45] |
| Various Chinese varieties (NS*) | EC (age NS*) | NS*                 | AGL1 (pDM805)                       | 30–40 min (2 d, 28°C)   | Timentin (150 mg/l) | Paromomycin          | 3.7–5.9             | 44                    | [46] |

IE – freshly isolated immature embryos; PCIE – pre-cultured immature embryos; EC – embryogenic callus; *NS not specified.
pre-cultured rice calli [29] but a plasmolysis step using 20% maltose failed to improve T-DNA delivery in 10 day pre-cultured wheat embryos [30].

**Agrobacterium tumefaciens strains and binary vectors**

The ability of particular *Agrobacterium* strains to transform plant cells is defined by their chromosomal and plasmid genomes which between them must encode all the machinery necessary for attachment and DNA-transfer. *The Agrobacterium* strains that have been successfully used for wheat transformation are based on only two chromosomal backgrounds, LBA4404 (Ach5) and C58 but these have been used with a wide range of Ti and binary plasmids. Some strains, notably AGL0 and AGL1 have been engineered to contain the so-called hypervirulent Ti plasmid, pTiBo542 harbouring additional vir genes originating from the *Agrobacterium* strain A281 which in its oncogenic form possesses a broad host range and a induces large, rapidly appearing tumours [31]. The strains used in the papers reviewed (see Table 2), also contain a binary and sometimes helper plasmids, often conferring yet more copies of virulence genes. A comparison of different *Agrobacterium* strains demonstrated that AGL0, a hypervirulent strain containing a disarmed pTiBo542 plasmid [32], was better at generating wheat transformants than other strains tested [19]. The ability of the Ti plasmid pTiBo542 to confer higher transformation efficiencies was first observed in dicots [33-35] and the vir genes from this plasmid have been widely adopted for monocot transformation vectors (reviewed by [11]). The weakly virulent *Agrobacterium* strain LBA4404, was successful in transforming wheat only when augmented by the superbinary plasmid pHK21 which possessed extra copies of vir B, C and G genes from pTiBo542 but not when carrying a standard binary plasmid [20]. Further evidence of the positive effect of additional vir genes was provided by the demonstration that a 15 Kb fragment of pTiBo542 on a pSOUP helper plasmid [36] enhanced T-DNA delivery and the production of transgenic wheat plants, even when in a hypervirulent Ach1 background already containing pTiBo542 as a resident Ti plasmid [21,37]. Although there has been a tendency to incorporate additional vir genes, particularly virG, into binary vectors this is not always necessary, at least for cv Bobwhite, in which a large number of transgenic lines have been reported using apparently standard *Agrobacterium* strains and binary vectors [16-18]. There is also one report [15] of transformation with a normal binary in the *Agrobacterium* strain C58C1 which the authors describe as disarmed, however it is our understanding that the C58C1 strain is actually cured of its pTiC58 plasmid [38,39]. There is currently insufficient data to define precisely which vir genes are necessary and where they should reside for optimal wheat transformation in different genotypes. There is also scope for further research into the effect on wheat trans-

formation of specific *Agrobacterium* mutants that have shown beneficial effects for other plant species. For example, strains containing mutations in the vir gene regulator virG resulting in constitutive expression of this gene and presumably the other vir genes it regulates, gave significant increases in efficiency of transformation in tobacco and cotton [40], *Catharanthus roseus* [41] and Norway spruce [42]. This virG mutant was also combined with a high copy number plasmid to further improve transformation rates in rice and soybean [43].

**Inoculation and co-cultivation**

The *Agrobacterium* infection process is divided into two stages: first, a short period, typically a few minutes to a few hours (see Table 1), of inoculation by complete or partial immersion of explants in an *Agrobacterium* suspension. Then, after the majority of *Agrobacterium* cells are removed by pouring or pipetting, the explants are co-cultivated for a further 1–3 days. One or both these steps are carried out in darkness at approximately 25 °C, although a two temperature co-cultivation step has also been tried with one day at 27 °C then two days to 25 °C [20]. During the cocultivation period, phenolic inducers such as acetylsyringone work alongside other signalling factors such as temperature and an acid environment to promote the expression of vir genes. The presence of 200 μM acetylsyringone in the *Agrobacterium* or co-cultivation medium markedly increased T-DNA delivery [21]. Enhanced transient GFP expression was observed in wheat cell clusters with acetylsyringone at 400 μM in the co-cultivation but not the inoculation media [19]. The need for acetylsyringone has been reported for a variety of wheat explants types [17,37,44] but not for wheat cell suspension cultures where exogenous induction agents were not necessary for stable transformation [17].

The use of surfactants during inoculation and co-cultivation significantly increases T-DNA delivery. Increasing concentrations of Silwet L-77 up to 0.04% had positive effects on T-DNA delivery as measured by the number of immature embryos with GUS foci and the number of GUS transient GFP expression was observed in wheat cell clusters with acetylsyringone at 400 μM in the co-cultivation but not the inoculation media [19]. The need for acetylsyringone has been reported for a variety of wheat explants types [17,37,44] but not for wheat cell suspension cultures where exogenous induction agents were not necessary for stable transformation [17].

**Control of Agrobacterium, regeneration and selection**

After the co-cultivation period, infected explants progress in a series of tissue culture steps on media designed to
inhibit the growth of Agrobacterium cells and promote regeneration and selection of transformants. The antibiotics used to control the growth of Agrobacterium are added immediately after co-cultivation during the callus induction phase and are maintained in all subsequent media. Timentin or carbenicillin are commonly used but other compounds such as cefotaxin, cefotaxime, ticarcillin and vancomycin have also been reported (see Table 1).

Plant selection agents complementary to the marker gene on the T-DNA are introduced to kill or compromise the growth of untransformed material. Selection for plant regeneration during the callus induction phase and are maintained in all subsequent media. Selection for plant regeneration during the callus induction phase and are maintained in all subsequent media.

### Concluding remarks

The advantages arising from simple molecular integrations of single copy DNA fragments with defined ends have driven research into Agrobacterium-mediated plant transformation. Compared to rice and maize, progress with wheat has been slower but as described here, robust methods for the transformation of wheat using Agrobacterium now exist. There is scope to further optimise the media components and pH and to investigate the ideal virulence gene complement. Current bottlenecks limiting throughput include the labour-intensive steps of embryo isolation and transfers between media. Unlike biolistics, Agrobacterium suspensions can be manipulated by liquid handing robots and this combined with the use of callus
cultures and the automation of transfer steps would enable a higher throughput which even at low efficiency would allow significantly more transgenic lines to be produced.

**A protocol for wheat (Triticum aestivum L.) transformation mediated by Agrobacterium tumefaciens**

**Scope and limitations**

This method was developed for the winter wheat cultivar Florida but with minor modifications has also been used to successfully transform the spring wheat varieties Fielder and Cadenza. It utilises the super-virulent *Agrobacterium tumefaciens* strain AGL1 [32] containing the plasmids pAL154/pAL156 which are based on the plasmid pSoup/pGreen [36], http://www.pGreen.ac.uk. The binary vector pAL156 contains a single T-DNA incorporating the bar gene conferring Basta resistance and a modified uidA (GUS) gene with an intron within the open reading frame to prevent its expression in *Agrobacteium* itself. Both the bar and uidA genes are driven by the maize ubiquitin1 promoter plus ubiquitin1 intron [49]. The bar gene is located next to the left border, and uidA is adjacent to the right border. A helper plasmid pAL154 provides replication functions for pAL156 in trans and also contains the 15 kb Komari fragment [35,47] supplying extra *vir* genes. Other *Agrobacterium* strains and plasmid combinations may also be appropriate in our protocol but have not yet been tested.

There are three main steps in the method: 1. incubation of freshly-isolated immature embryos with *Agrobacterium* *tumefaciens*; 2. induction of embryogenic callus and regeneration of shoots and roots; 3. application of a herbicide selection system to allow only the transgenic plantlets to survive. The average efficiency of transformation (number of independent transgenic lines/total number of immature embryos inoculated) is approximately 1%. The protocol takes 9–12 weeks from the isolation of immature embryos to the potting of putative transgenic plantlets to soil (Figure 1).

**Protocol**

**Growth of donor plants**

1.1 Sow seeds, 4–5 per 21 cm diameter pot, in compost which contains 75% fine-grade peat, 12% screened sterilised loam, 10% 6 mm screened lime-free grit, 3% medium vermiculite, 2 kg Osmocote Plus/m3 (slow-release fertiliser, 15N/11P/13K plus micronutrients), 0.5 kg PG mix/m3 (14N/16P/18K granular fertiliser plus micronutrients (Petersfield Products, Leicestershire, UK). Although other soil formulations may also be suitable.

1.2 Grow wheat plants in environmentally controlled growth rooms for approximately 11 weeks to provide immature seeds.

1.3 Growth rooms are maintained at 18–20°C day and 14–15°C night temperatures with a relative air humidity of 50–70% under a 16 h photo-period provided by banks of 400 W High Temperature Quartz Iodine lamps (Osram Ltd., Berkshire, UK) which give light intensity ~700 µmolm⁻²s⁻¹ photosynthetically active radiation (PAR).

1.4 Before transferring to these conditions, winter wheat varieties are vernalised from seed for 8 weeks at 4–5°C with a 12 hour photoperiod provided by 70 W fluorescent lamps giving approximately 150 µmolm⁻²s⁻¹ PAR at 300 mm from the lights.

1.5 The water is supplied by an automated flooding system, but seedling-stage plants are initially top watered individually for a few weeks [50].

**2 Growth and preparation of Agrobacterium cells for inoculation**

2.1 Initiate *Agrobacterium* liquid cultures by adding ~200 µl of a standard glycerol inoculum to 10 ml MG/L [51]
Table 3: Composition of medium MG/L

| Component          | /litre |
|--------------------|--------|
| Mannitol           | 5 g    |
| L-Glutamic acid    | 1 g    |
| KH₂PO₄             | 250 mg |
| NaCl               | 100 mg |
| MgSO₄·7H₂O         | 100 mg |
| Tryptone           | 5 g    |
| Yeast extract      | 2.5 g  |
| pH 7.0             |        |
| Biotin (added after autoclaving from stock at 1 mg/100 ml) | 1 µg |

(Add 100 µl to 1 litre MG/L)

3.2 Isolation of immature embryos

3.2.1 Isolate the embryos from the seed under a stereo microscope in a sterile environment using a sharp scalpel.

3.2.2 Remove and discard the embryo axis first then isolate the remaining portion of the embryo which is now referred to as the scutellum.

3.2.3 Plate scutella with the axis side (now removed) down onto semi-solid inoculation medium in 55 mm Petri dishes, about 50 scutella per plate.

3.2.4 It is important to inoculate each plate of 50 scutella with Agrobacterium tumefaciens, as described below, before isolating embryos for the next plate.

4 Inoculation of scutella with Agrobacterium tumefaciens

4.1 Take the resuspended Agrobacterium suspension from the shaker, add 60 µl 1% Silwet to make a final concentration of 0.015% and pour the whole 4 ml over a batch of 50 plated scutella.

4.2 Incubate for 1–3 hours at room temperature while preparing more scutella for inoculation as described in 3.2.

4.3 Transfer the scutella without blotting, keeping the ex-axis side down, onto fresh inoculation medium in 55 mm dishes. Allow to co-culture in the dark at 22–23 °C for 2–3 days.

5 Control of Agrobacterium and induction of embryogenic calli, regeneration and selection

5.1 After 2–3 days co-cultivation, transfer all scutella to induction medium (Table 4) and continue to incubate in the dark at 24–25 °C.

5.2 After 18 days, transfer embryogenic calli to RDZ medium (Table 4), and incubate at 24–25 °C but in the light. Embryogenic calli derived from the same immature embryo should be kept intact without breaking up.

5.3 After 3 weeks, transfer embryogenic calli to selection medium RPPT (or appropriate selection agent, Table 4). At this point, the calli can be broken into defined shoots/roots, but it is important to keep these together, or mark them clearly as there is possibility that these may be clones.

5.4 Continue transferring to fresh RPPT every 3 weeks until PPT tolerant plantlets are ready to be potted to soil.

Note, at the end of the first round of selection, some of the transgenic plants may be identified by GUS assay on leaf fragments. If they have good strong roots, they may be

Note, due to asynchronous development, only half or two thirds of the seeds on any one ear will be suitable, the seeds nearest to the peduncle are generally younger and smaller.
transferred to soil or put into the vernalisation room immediately, otherwise, transfer them to R medium without PPT for root strengthening (Table 4).

### 6 Materials

**6.1 Media for growing Agrobacterium tumefaciens**

See Table 3.

**6.2 Media for plant tissue culture**

6.2.1 Plant tissue culture media are prepared from stock solutions at double strength to allow the addition of an equal volume of gelling agent; Phytagel for inoculation and induction media, agar for RDZ, RPPT, and R media. Gelling agents are also prepared at double strength (Phytagel at 4 g/l and agar at 10 g/l) and autoclaved at 121°C for 20 min (see Table 4).

6.2.2 To make single-strength liquid inoculation media for resuspending Agrobacterium cells in section 2.3, simply mix double-strength medium with autoclaved, distilled water.

**Stock solutions for basal culture media**

Detailed below are the recipes for stock solutions of basal culture media components adapted from [50].

6.2.3 MS Macrosalts (×10):

| Component            | Inoculation (g/l) | Induction (g/l) | RDZ (g/l) | RPPT (g/l) | R (g/l) |
|----------------------|-------------------|----------------|-----------|------------|--------|
| MS Macro salts (×10) | 16.5 g/l NH₄NO₃   | 16.5 g/l NH₄NO₃| 16.5 g/l NH₄NO₃| 16.5 g/l NH₄NO₃| 16.5 g/l NH₄NO₃|
| L7 Micro salts (×1000) | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O |
| FeNaEDTA (×100)      | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O |
| MS vitamins (×1000)  | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O |
| Vitamins/Inositol (×200) | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  |
| Inositol             | -                 | -              | -           | -           | -       |
| Glutamine            | -                 | -              | -           | -           | -       |
| Casein hydrolysate   | -                 | -              | -           | -           | -       |
| MES                  | -                 | -              | -           | -           | -       |
| Glucose              | 200 mg            | 200 mg         | 200 mg      | 200 mg      | 200 mg  |
| Malate               | 200 mg            | 200 mg         | 200 mg      | 200 mg      | 200 mg  |
| pH adjusted to 5.8 then autoclaved | 0.1 mg          | -             | -           | -           | -       |
| 2,4-D                | 2 mg              | 0.5 mg         | 0.1 mg      | -           | -       |
| Picloram             | 2.0 mg            | 2.0 mg         | -           | -           | -       |
| Acetosyringone       | 200 µM            | -              | -           | -           | -       |
| Zeatin               | -                 | 160 mg         | 160 mg      | 160 mg      | 160 mg  |
| Zeatin               | -                 | 160 mg         | 160 mg      | 160 mg      | 160 mg  |
| PPT                  | -                 | -              | 2–4 mg      | 3–4 mg      | -       |

Note, Dissolve each component in distilled water separately before mixing. Autoclave at 121°C for 20 min and store at 4°C.

6.2.4 L7 Microsalts (×1000):

| Component            | Inoculation (g/l) | Induction (g/l) | RDZ (g/l) | RPPT (g/l) | R (g/l) |
|----------------------|-------------------|----------------|-----------|------------|--------|
| MS Macro salts (×10) | 16.5 g/l NH₄NO₃   | 16.5 g/l NH₄NO₃| 16.5 g/l NH₄NO₃| 16.5 g/l NH₄NO₃| 16.5 g/l NH₄NO₃|
| L7 Micro salts (×1000) | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O |
| FeNaEDTA (×100)      | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O |
| MS vitamins (×1000)  | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O | 15.0 g/l MnSO₄·7H₂O |
| Vitamins/Inositol (×200) | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  | 2.0 mg 200 g/l  |
| Inositol             | -                 | -              | -           | -           | -       |
| Glutamine            | -                 | -              | -           | -           | -       |
| Casein hydrolysate   | -                 | -              | -           | -           | -       |
| MES                  | -                 | -              | -           | -           | -       |
| Glucose              | 200 mg            | 200 mg         | 200 mg      | 200 mg      | 200 mg  |
| Malate               | 200 mg            | 200 mg         | 200 mg      | 200 mg      | 200 mg  |
| pH adjusted to 5.7 then filter sterilised | 0.1 mg          | -             | -           | -           | -       |
| 2,4-D                | 2 mg              | 0.5 mg         | 0.1 mg      | -           | -       |
| Picloram             | 2.0 mg            | 2.0 mg         | -           | -           | -       |
| Acetosyringone       | 200 µM            | -              | -           | -           | -       |
| Zeatin               | -                 | 160 mg         | 160 mg      | 160 mg      | 160 mg  |
| Zeatin               | -                 | 160 mg         | 160 mg      | 160 mg      | 160 mg  |
| PPT                  | -                 | -              | 2–4 mg      | 3–4 mg      | -       |

Note, MnSO₄ may have various hydrated states which will alter the required weight. For MnSO₄⋅H₂O, add 17.05 g/l, for MnSO₄⋅4H₂O, add 23.22 g/l, for MnSO₄⋅7H₂O,
add 27.95 g/l. Prepare 100 ml microsomal stock solution at a time. Filter sterilise, and store at 4°C.

6.2.5 MS Vitamins (-Glycine) (×1000):
0.1 g/l Thiamine HCl (Sigma-Aldrich),
0.5 g/l Pyridoxine HCl (Sigma-Aldrich),
0.5 g/l Nicotinic acid (Sigma-Aldrich).
Prepare 100 ml at a time. Filter sterilise, and store at 4°C.

6.2.6 Vitamins/Inositol (×200):
40.0 g/l Myo-Inositol (Sigma-Aldrich),
2.0 g/l Thiamine HCl (Sigma-Aldrich),
0.2 g/l Pyridoxine HCl (Sigma-Aldrich),
0.2 g/l Nicotinic acid (Sigma-Aldrich),
0.2 g/l Ca-Pantothenate (Sigma-Aldrich),
0.2 g/l Ascorbic acid (Sigma-Aldrich).
Filter sterilise and store at -20°C in 10 ml aliquots.

6.2.7 Supplements
- Acetosyringone (3’,5’-Dimethoxy-4’-hydroxyacetophenone) (Aldrich D12,440-6: MW-196.20), Dissolve in 70% ethanol to give 10 mg/ml or 50 mM stock solution. Filter sterilise, aliquot and store at -20°C.
- 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich), 1 mg/ml in ethanol/water (dissolve powder in ethanol then add water to volume). Filter sterilise, and store at -20°C in 1 ml aliquots.
- Zeatin mixed isomers (10 mg/ml) (Sigma-Aldrich), Dissolve powder in small volume 1 M HCl and make up to volume with water, mix well/vortex. Filter sterilise, and store at -20°C in 1 ml aliquots.
- Picloram (1 mg/ml) (Sigma-Aldrich), Dissolve picloram in water, filter sterilise and store at -20°C in 2 ml aliquots.
- Timentin (300 mg/ml) (Melford, UK), Dissolve Timentin (Ticarcillin/Clavulanic (15:1)) in water, filter sterilise and store at -20°C in 1 ml aliquots.
- PPT (10 mg/ml) (Glufosinate Ammonium) (Melford, UK), Dissolve in water, mix well/vortex, filter sterilise, and store at -20°C in 1 ml aliquots.
- Silwet L-77 (1% v/v) (Lehle seeds, USA), Dissolve in water, filter sterilise, and store at 4°C in 0.5 ml aliquots.

Competing interests
The author(s) declare that they have no competing interests.

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