Utilization of sucrose during cocultivation positively affects *Agrobacterium*-mediated transformation efficiency in sugar beet (*Beta vulgaris* L.)

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Abstract: Sugar beet (*Beta vulgaris* L.) is one of the most important industrial crops throughout the world. With the availability of suitable genetic transformation technologies, the yield, quality, and stress tolerance of sugar beet could be improved significantly. However, low transformation efficiencies seriously limit the application of molecular technologies to the genetic improvement of sugar beet. With the aim of improving gene transfer techniques for sugar beet, the effect of different sucrose concentrations during cocultivation on the initial *Agrobacterium*-mediated transformation efficiencies in sugar beet was tested. To develop an efficient experimental system through which the effect of sucrose could be tested, first, a prolific regeneration system was optimized by testing the effect of different plant growth regulators on in vitro regeneration and rooting efficiencies from sugar beet cotyledonary node explants. The highest mean number of regenerated shoots per explant was obtained when the cotyledonary node explants excised from young seedlings were grown on MS medium supplemented with 1.0 mg/L 6-benzylaminopurine. Using this regeneration system, the effect of different concentrations of sucrose included in the cocultivation medium on the initial genetic transformation efficiencies observed in T₀ plants was tested using an *Agrobacterium tumefaciens* strain carrying the pBin19/35S:GUS-INT construct. The inclusion of 4.5% sucrose in the cocultivation medium resulted in significantly higher transformation (34.09%) and expression efficiencies (22.72%), confirmed by polymerase chain reaction and β-glucuronidase assays, respectively, in regenerated T₀ seedlings. If translated into stably inherited transformation efficiencies, these findings could contribute to the success of genetic transformation studies in sugar beet and other crops recalcitrant to *Agrobacterium*-mediated transformation.

Key words: Sugar beet, genetic transformation, cocultivation, plant growth regulators

1. Introduction

Industrial crops are cultivated annual or perennial plants that are used as raw material by various branches of industry and grow in various geographic regions including temperate, subtropical, and tropical latitudes (Vijayan et al., 2008; Erçilsi et al., 2011; Ahmad et al., 2015; Cesur et al., 2018).

Sugar beet is one of the most important industrial plants widely cultivated in temperate climates of the world (Mishutkina et al., 2010; Lytvyn et al., 2014), and it is produced in 55 countries, with major producing countries being Russia, France, the USA, Germany, and Turkey. A number of biotic and abiotic stresses cause significant crop and quality losses in sugar beet (Lytvyn et al., 2014). Genetic transformation technologies offer great promise for improving stress tolerance in sugar beet and other crop plants through the introduction of genes conferring biotic and abiotic tolerance. Although a number of different techniques have been developed for the genetic transformation of plants, *Agrobacterium*-mediated transformation is one of the most efficient and thus commonly used plant transformation methods. However, low transformation efficiencies remain a limiting factor in *Agrobacterium*-mediated genetic transformation of many crop plants. For example, despite the fact that the first successful sugar beet transformation was achieved at the end of the 20th century, low transformation and regeneration efficiencies still hinder the routine production of transgenic sugar beet plants (Mishutkina et al., 2010; Rivera et al., 2012; Pathi et al., 2013).

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A number of variables affect Agrobacterium-mediated transformation efficiencies in plants, including the Agrobacterium strain, the type and concentrations of plant selectable markers, cocultivation time and bacterial concentrations, and the pH and composition of the media used for plant regeneration, as well as the promoters used to drive the expression of transgenes (Pathi et al., 2013). Transformation rates can also be influenced by T-DNA transfer efficiencies from Agrobacterium to plant cells, which, in turn, can be dependent on sufficient levels of Vir gene activation (Stachel and Zambryski, 1986; Yanofsky et al., 1986; Godwin et al., 1991). Vir gene induction is known to be affected by various factors, such as phenolics (e.g., acetosyringone), sugars, temperature, plant growth regulators, and the pH of the cocultivation medium (Jin et al., 2005; Uranbey et al., 2005; Kumlehn et al., 2006; Opabode, 2006).

The aim of this study was to test the effect of sucrose used during the cocultivation stage on the initial Agrobacterium-mediated transformation efficiencies observed in T₀ sugar beet plants. First, an improved in vitro regeneration method for sugar beet was developed, and then the effect of different sucrose concentrations used in the cocultivation medium on the initial transformation efficiencies was tested. The results revealed that 4.5% sucrose led to a significant increase in the efficiency of transformed T₀ sugar beet seedlings.

2. Materials and methods

2.1. Surface sterilization of the seeds
Sugar beet seeds (cultivar Pauletta) supplied by the Sugar Institute (Ankara, Turkey) were surface sterilized using 30% commercial bleach containing a few droplets of Tween 20 for 30 min followed by three 5-min rinses with sterile water.

2.2. In vitro plant regeneration
The sterilized sugar beet seeds were germinated on MS medium (Murashige and Skoog, 1962) containing 4.43 g/L MS salts (Caisson Labs, Smithfield, UT, USA), 30 g/L sucrose, and 4 g/L agar (Duchefa Biochemie, Haarlem, the Netherlands) at 24 °C in the dark for 3 days. The germinated seedlings were incubated in a growth chamber with a light/dark photoperiod of 16:8, at a constant temperature of 24 ± 2 °C, and irradiated at 50 ± 5 µmol m⁻² s⁻¹ with cool white fluorescent tubes (Master TL-D 840, Philips, Pila, Poland) at a relative humidity of 70 ± 10%. The regeneration efficiencies were evaluated after 3–4 weeks.

2.3. In vitro rooting and acclimatization
Well-developed shoots were transferred to rooting medium after 3–4 weeks of incubation on the regeneration medium. The rooting medium contained 2.2 g/L MS salts, 30 g/L sucrose, and 5 g/L plant agar with varying concentrations of indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.00, 0.50, 1.00, 1.50, or 2.00 mg/L). Plants with well-developed roots were transplanted into small containers with a 2:1 sterilized peat:perlite mix and allowed to grow further. Young seedlings were transferred to pots, covered with transparent bags, and incubated at 50% humidity at 25 °C until well-developed regenerants were obtained.

2.4. Agrobacterium-mediated gene transfer
The Agrobacterium strain GV2260 carrying the binary vector pBIN19/35S-GUS-INT (Vancanneyt et al., 1990), kindly provided by Ankara University (Ankara, Turkey), was used in the gene transfer experiments. The Agrobacterium strain was grown by shaking in liquid LB containing 50 mg/L kanamycin and 20 mg/L rifampicin. The density of the culture was adjusted to OD₆₀₀ of 0.6–0.7 before the inoculations. For cocultivation, cotyledonal node explants (1 cm in length) taken from seedlings grown in vitro were immersed in the Agrobacterium culture for 30 min. The treated explants were then moved onto cocultivation plates containing MS salts (4.4 g/L), varying concentrations of sucrose (1.5%, 3%, 4.5%, 6%, and 9%), and 100 µM acetosyringone and incubated at 25 °C for 72 h. After cocultivation, the explants were transferred to regeneration medium containing 4.44 g/L MS salts, 30 g/L sucrose, 1 mg/L BAP, 5 g/L plant agar, and 50 mg/L kanamycin. Next, 1 mL/L Duocid (ampicillin) (Pfizer, Latina, Italy) was also added to the regeneration medium to prevent bacterial growth.

2.5. Molecular analyses
DNA isolated from the putatively transformed plants, using the methods of Lefort et al. (1998), was quantified using a NanoDrop ND-1000 spectrophotometer and run on 1% agarose gel to check its integrity. To confirm the transgenic status of the treated tissues, polymerase
chain reaction (PCR) analyses were performed to detect the presence of the NPTII gene using the following primer sequences: (forward) 5’-ttgctcctgccgaaaa-3’ and (reverse) 5’-gaaggcgataagaagccga-3’, designed using UniproUGENE v.1.240 software according to the methods of Okonechnikov et al. (2012). PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc., Singapore) using the GoTaq G2 Flexi DNA polymerase enzyme in the standard PCR reactions. The PCR conditions comprised an initial cycle of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 2 min at 72 °C with a final extension for 10 min at 72 °C.

2.6. GUS assays
To determine the transformation status, histochemical β-glucuronidase (GUS) staining was conducted on the putatively transformed sugar beet plants. Tissue samples taken from the T0 plants were incubated in the GUS staining solution containing 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, and 1 mM X-Gluc at 37 °C overnight and were cleared using 70% ethanol.

2.7. Statistical analysis
All of the statistical analyses were done using the IBM SPSS Statistics 22.0 program (IBM Corp. Armonk, NY, USA) according to the randomized plot design. Statistically significant values were determined by ANOVA followed by the Tukey post hoc test.

3. Results
3.1. Optimization of an in vitro plant regeneration system for sugar beet to test transformation efficiencies
To develop an efficient regeneration system that could be used for the optimization of sugar beet transformation, cotyledonary node explants taken from sugar beet seedlings grown in sterile conditions were treated with different concentrations of BAP, GA3, or TDZ (Figure 1). For GA3, the highest regeneration rates (5.05 and 4.60 regenerants/explant) were obtained with concentrations of 1.0 mg/L and 1.5 mg/L GA3, respectively, while the other GA3 concentrations tested (2.00, 2.50, and 3.00 mg/L) produced relatively low regeneration rates (3.20, 2.80, and 2.65 regenerants/explant, respectively) (Figure 2). Different concentrations of TDZ (0.5–3 mg/L) did not significantly affect the regeneration rates (Figure 2).

We also tested the effect of different concentrations of plant growth regulators on the average shoot length per explant. In these experiments, the longest shoot lengths (average 4.26 cm length/explant) were obtained from the regenerants grown on medium containing 2.50 mg/L GA3. An average shoot length of 3.25 cm was attained when 0.5 mg/L BAP was used in the medium, while the different concentrations of TDZ tested did not have any significant effect on shoot length (Figure 3). Regenerated seedlings were subsequently transferred to rooting medium containing different concentrations of either IBA or IAA. The rooting medium containing 1 mg/L IAA produced the highest number of roots per explant, while the medium containing 2 mg/L IAA promoted root elongation (Figures 4a and 4b). Different concentrations of IBA used in the rooting medium did not significantly affect the average root number or length.

3.2. Effect of sucrose used during cocultivation on the initial transformation efficiencies
We tested the effect of different concentrations of sucrose added to cocultivation media on transformation efficiency after PCR analysis and GUS staining (Figure 5). Our analyses revealed a significant effect of the sucrose concentrations used during cocultivation on the T0 transformation efficiencies in sugar beet. The explants cocultivated in medium containing 4.5% sucrose produced the highest transformation efficiency (34.09%) based on the percentages of NPTII-positive T0 seedlings and the highest expression efficiency based on the percentages of GUS-positive (22.72%) T0 plantlets produced (Table).

4. Discussion
Successful applications of plant biotechnology to agriculture by transferring useful genes have great potential for improving the quality and stress tolerance of crop plants. In recent years, significant progress has been made in developing gene transfer technologies for a wider number of crop plants using Agrobacterium. As an industrial crop, sugar beet acquires potential benefits from biotechnological applications, including genetic transformation technologies that are efficient and reproducible. To achieve this, the development of an improved regeneration and transformation system is required. In this study, we aimed to develop an efficient regeneration system that could facilitate the high-frequency transformation of sugar beet using Agrobacterium.

In this study, 1–1.5 mg/L BAP produced the highest regeneration rates. Similarly, Ritchie et al. (1989), Mishutkina and Gaponenko (2006), and Ergül et al. (2018) reported a significantly positive effect of 1–2 mg/L, 0.5–1 mg/L, and 1–3 mg/L BAP, respectively, on the regeneration rates of wild and cultivated sugar beet plants. Slightly different concentrations of BAP were found to be beneficial for regeneration rates in different studies, which might be due to the differences of the sugar beet genotypes used in each study. Nevertheless, it is evident that different studies have demonstrated the importance of cytokinins on sugar beet regeneration rates from cotyledonary node explants.
Figure 1. In vitro regeneration and acclimatization of sugar beet. Transfer of cotyledonary node explants onto shoot regeneration medium (A). Regenerated shoots were subcultured (B) and developing shoots were transferred onto the rooting medium (C). Putative T₀ plantlets showing well-developed roots (D) were transferred to a growth chamber for acclimatization (E).

Figure 2. The effects of BAP, GA₃, and TDZ on sugar beet shoot regeneration expressed as average shoot number ± SE per explant. Different letters indicate statistically significant differences between the treatments (Tukey’s HSD, P < 0.05).
In this study, the highest root formation rate was observed on medium supplemented with 1 mg/L IAA, while 2 mg/L IAA promoted root length better than 1 mg/L IAA. Our results on rooting efficiencies might differ slightly from other reports (Norouzi et al., 2005; Jafari et al., 2009; Ergül et al., 2018), presumably due to the composition of the media and genotypic differences.

In the regeneration and transformation studies reported here, we used cotyledonary node explants that may have contained preexisting meristems. However, it is likely that the shoots we obtained within 3–4 weeks were likely formed through a de novo regeneration process. Indeed, Krens et al. (1996) reported that regeneration from preexisting meristems takes place more rapidly than
de novo regeneration (1 week vs. 3–4 weeks) in sugar beet. Therefore, these authors considered shoots forming after 3 weeks of in vitro culture as the products of de novo regeneration.

The cotyledonary node explant-based transformation system has also been used by other researchers for sugar beet transformation (Lindsey and Gallois, 1990; Konwar, 1994). For instance, Lindsey and Gallois (1990) reported that cotyledonary node explants were more suitable for sugar beet transformation than leaf explants due to the ability of the latter to rapidly produce new shoots without an intermediary callus phase. Regeneration from cotyledonary node explants was also rapid and took place within 3–4 weeks in our study. Mishutkina and Gaponenko (2006) reported direct regeneration from cotyledonary node explants within 4–6 weeks. In contrast, 15–20 weeks were required to regenerate plantlets through a callus phase from other explant types. The direct regeneration of transformed cells from cotyledonary node explants may prevent the somaclonal variation that was often observed in callus-based transformation methods (Hisano et al., 2004; Ergül et al., 2018).

Figure 4b. The effect of IBA and IAA on average root length (±SE) per shoot. Different letters indicate statistically significant differences between the treatments (Tukey’s HSD, P < 0.05).

Figure 5. Confirmation of transformation status by histochemical GUS assays (A) and by amplifying the NPTII gene from genomic DNA samples isolated from the leaves of putatively transformed T$_0$ plants by PCR (B). NC and PC indicate negative and positive controls, respectively.
The availability of an efficient regeneration system enabled the testing of the effect of different sucrose concentrations on transformation efficiencies. Using this regeneration system, the effect of different sucrose concentrations on the efficiency of gene transfer by \textit{Agrobacterium} was tested herein. The results demonstrated a positive effect of 4.5% sucrose transformation efficiency, as determined by the analysis of T\textsubscript{0} plantlets that contained the \textit{NPTII} gene conferring resistance to kanamycin. These plantlets were further analyzed for the expression of the \textit{GUS} transgene. It should be noted that the \textit{GUS} gene in the 35S:GUS-INT construct contained an intron that prevented the expression of \textit{GUS} in \textit{Agrobacterium} (Vancanneyt et al., 1990). The reason why the 4.5% sucrose concentration was more efficient than the other tested sucrose concentrations was unknown. However, it is well established that \textit{Agrobacterium Vir} gene activation is dependent on various medium components that include phenolic compounds such as acetosyringone and sugars. Indeed, in the absence of acetosyringone during cocultivation, transformation efficiencies are significantly reduced (Kumlehn et al., 2006; Opabode, 2006). Although it is unknown if the increased transformation efficiencies observed here resulted from increased \textit{Vir} gene activation, the effect of sugars on \textit{Vir} gene activation has been previously demonstrated (Stachel and Zambryski, 1986; Yanofsky et al., 1986; Shimoda et al., 1990; Godwin et al., 1991). In addition, it was shown that ChvE, encoding a chromosomally located virulence protein in \textit{Agrobacterium}, binds several neutral sugars and interacts with at least 2 \textit{Vir} gene products to stimulate virulence (Hu et al., 2013). Furthermore, Alt-Mörbe et al. (1989) reported that the continuous induction of the \textit{VirD} and \textit{VirE} genes of \textit{Agrobacterium} required at least 2% sucrose. The results reported here indicate the effect of a relatively higher sucrose concentration in mediating higher transformation efficiencies. This could be due to the use of a different \textit{Agrobacterium} strain in our study. Although T\textsubscript{1} and T\textsubscript{2} progeny from the T\textsubscript{0} plants were not examined, it was expected that the increased initial transformation efficiencies obtained would lead to improved rates, by which stably transformed sugar beet plants could be obtained.

In the transformation procedure used, it was observed that the percentage of kanamycin-resistant plantlets regenerated on 50 mg/L kanamycin-containing medium was much higher than those shown to contain the \textit{NPTII} gene by PCR, suggesting that at least a certain portion of the regenerants were escapes. This indicated that the kanamycin concentration used was not completely inhibitory on the regeneration of untransformed cells and this may have contributed to the relatively high percentage of plants that escaped from kanamycin selection, as was also reported by Norouzi et al. (2005). Indeed, Jafari et al. (2009) reported that concentrations of 50–75 mg/L kanamycin worked well in the initial stages of selection, while up to 100 mg/L kanamycin was needed to select transformed sugar beet plants at later stages of the transformation process. Similarly, Norouzi et al. (2005) used 150 mg/L kanamycin in the initial stages of sugar beet transformation. Nevertheless, kanamycin concentrations are often reduced to 50 mg/L in the rooting medium to avoid the inhibitory effects of kanamycin on root formation (Konwar, 1994; Norouzi et al., 2005). Therefore, we recommend that kanamycin concentrations should be carefully examined in order to minimize the number of potential escapes.

In conclusion, in this study, an in vitro regeneration protocol and a cocultivation procedure that increased the \textit{Agrobacterium}-mediated gene transfer efficiency in T\textsubscript{0} plants were optimized for sugar beet. Successful shoot regeneration from cotyledonary node explants was achieved and the effects of different plant growth regulators on this process were determined. Improved shoot and root regeneration was achieved on media containing 1

| Sucrose % (g/L) | Total number of explants used and (%) | Number of kanamycin resistant seedlings / (%) | Transformation efficiency number of \textit{NPTII} positive T\textsubscript{0} seedlings / (%) | Expression efficiency number of \textit{GUS} positive T\textsubscript{0} seedlings / (%) |
|----------------|----------------------------------------|---------------------------------------------|-------------------------------------------|---------------------------------------------|
| 1.5 (15)       | 44 / (100)                             | 11 / (25)bc                                | 3 / (6.81)b                               | 2 / (4.54)c                               |
| 3 (30)         | 44 / (100)                             | 19 / (43.18)b                              | 6 / (13.63)b                              | 4 / (9.09)c                               |
| 4.5 (45)       | 44 / (100)                             | 30 / (68.18)b                              | 15 / (34.09)b                             | 10 / (22.72)c                             |
| 6 (60)         | 44 / (100)                             | 25 / (56.81)ab                             | 8 / (18.18)ab                             | 6 / (13.63)b                              |
| 9 (90)         | 44 / (100)                             | 13 / (29.54)bc                             | 5 / (11.36)b                              | 4 / (9.09)c                               |

*Different letters indicate that the means are statistically significantly different (P < 0.05).
mg/L BAP and 1 mg/L IAA, respectively. In addition, the inclusion of 4.5% sucrose in the cocultivation medium had a significant positive effect on genetic transformation frequencies in T₀ sugar beet plants. It should be noted, however, that because sugar beet is a biennial plant species (i.e. takes 2 years to produce seed), we could not examine the transmission of transgenes into subsequent generations in this study. However, the increased transformation efficiency observed in generating the initial transformants (T₀) is expected to lead to increased rates of transgenic plants that transmit transgenes to their progeny in a stable manner. Therefore, it is expected that the results presented here will contribute to sugar beet biotechnology by improving the production of transgenic sugar beet lines.

References

Ahmad S, Raza I, Muhammad D, Ali H, Hussain S, Doğan H, Zia-Ul-Haq M (2015). Radiation, water and nitrogen use efficiencies of *Gossypium hirsutum* L. Turk J Agric For 39: 1-13.

Alt-Mörbe J, Kühmann H, Schröder (1989). Differences in induction of Ti-plasmid virulence genes virG and virD and continued control of virD expression by four external factors. Mol Plant Mic In 2: 301-308.

Cesur C, Eryılmaz T, Uskutoglu T, Doğan H, Cosge Şenkal B (2018). Cocklebur (*Xanthium strumarium* L.) seed oil and its properties as an alternative biodiesel source. Turk J Agric For 42: 29-37.

Ercisli S, Ipek A, Barut E (2011). SSR marker-based DNA fingerprinting and cultivar identification of olives (*Olea europaea*). Biochem Genet 49: 555-561.

Ergül A, Khabbazi S, Oğuz MÇ, Özmen CY, Gürel S, Gürel E (2018). In vitro multiplication of wild relatives in genus Beta conserves the invaluable threatened germplasms. Plant Cell Tiss Organ Cult 134: 169-175.

Jin S, Zhang X, Nie Y, Guo X, Huang C (2005). Factors affecting transformation efficiency of embryogenic callus of upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens*. Plant Cell Tiss Organ Cult 81: 229-237.

Konwar BK (1994). *Agrobacterium* tumefaciens-mediated genetic transformation of sugar beet (*Beta vulgaris* L.). J Plant Biochem Biotech 3: 37-41.

Krens FA, Trifonova A, Keizer LP, Hall RD (1996). The effect of exogenously-applied phytohormones on gene transfer efficiency in sugarbeet (*Beta vulgaris* L.). Plant Sci 116: 97-106.

Kumlehn J, Serazetdinova L, Hensel G, Becker D, Loerz H (2006). Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgegetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol J 4: 251-261.

Lefort F, Lally M, Thompson D, Douglas G (1998). Morphological traits, microsatellite fingerprinting and genetic relatedness of a stand of elite oaks (*Q. robur* L.) at Tullynally, Ireland. Silvae Genet 47: 257-261.

Lindsey K, Gallois PJ (1990). Transformation of sugarbeet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. J Exp Bot 41: 529-536.

Lytvyn D, Syvura V, Kurylo V, Olenieva V, Yemets A, Blume YB (2017). Creation of transgenic sugar beet lines expressing insect pest resistance genes cry1C and cry2A. Tisitol Genet 48: 69-75.

Mishutkina YV, Galaponenko A (2006). Sugar beet (*Beta vulgaris L.*) morphogenesis in vitro: effects of phytohormone type and concentration in the culture medium, type of explants, and plant genotype on shoot regeneration frequency. Genetika 42: 150-157.

Mishutkina YV, Kamionskaya A, Skryabin K (2010). The creation of sugar beet transgenic plants expressing bar gene. Appl Biochem Microbiol 46: 80-86.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Norouzi P, Malboobi MA, Zamani K, Yazdi-Samadi H (2005). Using a competent tissue for efficient transformation of sugarbeet (*Beta vulgaris* L.). In Vitro Cell Dev Biol-Plant 41: 11-16.

Okonechnikov K, Golosova O, Furfey M (2012). UniproUGENE: A unified bioinformatics toolkit. Bioinformatics 28: 1166-1167.

Opabode JT (2006). *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. Biotechnol Mol Biol Rev 1: 12-20.

Pathi KM, Tula S, Tuteja N (2013). High frequency regeneration via direct somatic embryogenesis and efficient *Agrobacterium*-mediated genetic transformation of tobacco. Plant Signal Behav 8: e24354.

Ritchie G, Short K, Davey MR (1989). In vitro shoot regeneration from callus, leaf axils and petioles of sugar beet (*Beta vulgaris* L.). J Exp Bot 40: 277-283.

Rivera AL, Gomez-Lim M, Fernandez F, Loske AM (2012). Physical methods for genetic plant transformation. Phys Life Rev 9: 308-345.

Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y, Machida, Y (1990). Control of expression of *Agrobacterium Vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. P Natl Acad Sci USA 87: 6684-6688.
Stachel SE, Zambryski P (1986). \textit{VirA} and \textit{VirG} control the plant-induced activation of the T-DNA transfer process of \textit{A. tumefaciens}. Cell 46: 325-333.

Uranbey S, Sevimay C, Kaya M, Ipek A, Sancak C, Basalma D, Er C, Ozcan S (2005). Influence of different co-cultivation temperatures, periods and media on \textit{Agrobacterium tumefaciens}-mediated gene transfer. Biol Plantarum 49: 53-57.

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990). Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in \textit{Agrobacterium}-mediated plant transformation. Mol General Genet 220: 245-250.

Vijayan K, Chakraborti SP, Ercisli S, Ghosh PD (2008). NaCl induced morpho-biochemical and anatomical changes in mulberry (\textit{Morus} spp.). Plant Growth Reg 56: 61-69.

Yanofsky MF, Porter SG, Young C, Albright LM, Gordon MP, Nester EW (1986). The \textit{virD} operon of \textit{Agrobacterium tumefaciens} encodes a site-specific endonuclease. Cell 47: 471-477.