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Tissue Culture and Regeneration of Three Rose Cultivars

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Abstract. **Methods of in vitro regeneration protocols were developed for three elite rose cultivars, Chewnicebell (Oso Easy Italian Ice®), Bucbi (Carefree Beauty™), and Cheweyesup (Ringo All-Star™). We evaluated the effects of different types and concentrations of auxins [dichlorophenoxyacetic acid (2,4-D) and trichlorophenoxyacetic acid (2,4,5-T)], carbohydrates [sucrose, glucose, and fructose], and cytokinins [thidiazuron (TDZ) and 6-bezylaminopurine (BAP)] on callus induction and regeneration from leaf explants. The greatest amount of regenerative callus was obtained on media containing 10 μM 2,4-D and 30 g·L^{-1} sucrose for Italian Ice® (40%), 10 μM 2,4-D and 60 g·L^{-1} glucose for Carefree Beauty™ (24%), and 5 μM 2,4,5-T and 30 g·L^{-1} sucrose for Ringo All-Star™ (32%). The greatest regeneration occurred when callus was transferred to media consisting of 1/2 MS media supplemented with 2.9 μM GA_3 and 5 μM TDZ for Italian Ice® and Ringo All-Star™, and with 2.9 μM GA_3 and 20 μM TDZ for Carefree Beauty™. Plantlets regenerated from callus were cultured on maintenance media and successfully transferred ex vitro. This study highlights the genotype-specific responses among rose cultivars and provides the first reports of in vitro regeneration for Italian Ice® and Ringo All-Star™.**

Rose (*Rosa ×hybrida*) is one of the world's most important ornamental crops with widespread commercialization across the landscape, floral, and fragrance industries. Rose breeders have developed many new varieties with desirable qualities such as compact size, recurrent flowering, and disease resistance (Byrne, 2015). However, rose breeding is tedious and time-consuming because modern cultivars are highly heterozygous with varying ploidy levels and complex genetic relationships among species and cultivars. This diversity results in barriers to sexual reproduction and erratic seed germination that complicates the breeding process (Gudin, 2000; Jacob et al., 1995; Rowley, 1966). Thus, introgressing genes of interest from different cultivars or wild species often requires multiple generations and can be challenging for many breeders (Debener et al., 1996; Zlesak, 2006).

Genetic transformation has the potential to improve several valuable traits quickly, including vase life, disease resistance, fragrance, plant form, and petal color. Most current transformation systems require an in vitro regeneration step. The first reports of in vitro regeneration in rose were published in the early 1990s (de Wit et al., 1990; Kunitake et al., 1993; Roberts et al., 1995). This process can be particularly limiting for rose due to the high heterozygosity and recalcitrant nature of many rose cultivars (Burrell et al., 2006). Multiple studies have reported a significant genotype-dependent response for rose regenerative callus production (Hsia and Korban, 1996; Kintzios et al., 1999; Nguyen et al., 2020; Yokoya et al., 1995).

Auxin source and concentration can play a significant role in regenerative callus production in rose. A synthetic analog of auxin indole-3-acetic acid (IAA), 2,4-dichloropenoxyacetic acid (2,4-D) has been used most extensively in the published reports of rose regeneration, especially through somatic embryogenesis (Shen et al., 2016). Other frequently used auxins for rose regenerative callus production include α-naphthalene acetic acid (NAA; Vergne et al., 2010), dicamba (Kim et al., 2004), p-chlorophenoxyacetic acid (Kintzios et al., 1999), and 2,4,5-trichlorphenoxyacetic acid (2,4,5-T; Estabrooks et al., 2007). 2,4,5-T is another synthetic analog of IAA and has been reported to have a weaker mutagenic effect than 2,4-D in yeast and mammalian cells (Venkov et al., 2000). This effect could be useful to retain regenerative callus production while minimizing somaclonal variation. 2,4,5-T has also been reported to increase embryogenic callus induction, somatic embryogenesis, and regenerated plantlet survival in 'Livin' Easy' rose (Estabrooks et al., 2007). Although these results were promising, no other studies using 2,4,5-T for rose regeneration have been published.

Carbohydrate source plays a significant role in the regeneration process. Glucose, fructose, and sucrose were used to generate mature somatic embryos in 'Trumpeter', 'Dr. Huey', and 'Tineke' roses (Castillon and Kamo, 2002). Glucose has been reported to produce more embryogenic callus than sucrose in Carefree Beauty™ rose (Hsia and Korban, 1996). Kunitake et al. (1993) found that the presence of fructose and glucose initiated the most regeneration in seed-derived callus of *R. rugosa*. Although carbohydrates have occasionally been tested for their effect on tissue culture and embryogenesis, no study has explored the effects of various concentrations of multiple carbohydrates on embryogenic callus initiation in rose.

Cytokinin source plays a significant role in regeneration in rose. Some commonly used cytokinins for rose regeneration include zeatin (Ludwig et al., 2000; Vergne et al., 2010; Zakizadeh et al., 2008), kinetin (Cai et al., 2022; de Wit et al., 1990), 6-benzylaminopurine (BAP; Hsia and Korban, 1996), and thidiazuron (TDZ; Cai et al., 2022; Chen et al., 2014; Li et al., 2002; Zakizadeh et al., 2008). BAP is a synthetic cytokinin that stimulates growth and cell division. It is widely used in tissue culture to promote axillary shoot proliferation and regeneration, and is commonly used for rose micropropagation (Katsumoto et al., 2007; Kim et al., 2004; Xing et al., 2010). TDZ was first used as a cotton defoliant in 1976 (Arndt et al., 1976) and has been used in tissue culture since 1988 (Lu, 1993). TDZ is one of the most potent cytokinin-like compounds for woody plant tissue culture (Huetteman and Preece, 1993).

The objectives of this study were to investigate the type and concentration of auxins, carbohydrates, and cytokinins on callus induction and regeneration through organogenesis or somatic embryogenesis for the Italian Ice®, Carefree Beauty™, and Ringo All-Star™ roses.

Materials and Methods

Plant materials and culture conditions. In vitro cultures of 'Chewnicebell' (Oso Easy Italian Ice®), 'Bucbi' (Carefree Beauty™), and 'Cheweyesup' (Ringo All-Star™) roses were initiated from apical and axillary bud explants. Rose genotypes were chosen based on their desirable ornamental characteristics. Explants were collected from containerized

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greenhouse-maintained plants and washed for 4 h under running water before being surface-sterilized in 20% v/v commercial bleach (6.25% NaOCl) containing one to two drops of Tween® 20. Explants were agitated for 20 min followed by three 5-min rinses in sterile distilled water. Explants were cultured in 180-mL glass jars containing 25 mL of shoot proliferation/maintenance media consisting of full-strength MS (Murashige and Skoog, 1962) salts and vitamins (PhytoTech Laboratories, Lenexa, KS, USA), 5 μM BAP, 0.5 μM NAA, 30 $g \cdot L^{-1}$ sucrose, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.1 $g \cdot L^{-1}$ 2-(N-morpholino) ethanesulfonic acid (MES), and 6.5 $g \cdot L^{-1}$ agar (A296; PhytoTech Laboratories, Lenexa, KS, USA). Medium pH was adjusted to 5.75 using 1 mM NaOH or HCl and autoclaved at 121 °C for 20 min. All explants were cultured under 16/8 h light/dark photoperiod (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lights) at ~23 °C. Proliferating microshoots were used as stock cultures for all experiments and maintained by transferring onto fresh media (25 mL in 180-mL glass jars) every 4 to 5 weeks.

Callus induction—auxin response. The effects of 2,4-D and 2,4,5-T at 5, 10, 20, or 40 μM concentrations on callus induction were investigated. Vigorously growing leaflets were excised from proliferating shoot cultures of the three cultivars and used for callus induction. Leaflets were scored across the midrib and placed abaxial side down in petri dishes (90 mm in diameter) containing 25 mL of callus induction media. Basal medium for all experiments consisted of full-strength MS salts and vitamins, 3% sucrose, 0.1 $g \cdot L^{-1}$ MES, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.75 $g \cdot L^{-1}$ $MgSO_4$, solidified with 2.5 $g \cdot L^{-1}$ Gelzan and supplemented with auxin treatment combinations. Media was adjusted to a pH of 5.75 before autoclaving. Explants were incubated in the dark for 4 weeks before being transferred to a regeneration media, based on Li et al. (2002) consisting of half-strength MS salts with full-strength vitamins, 3% sucrose, 0.1 $g \cdot L^{-1}$ MES, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.75 $g \cdot L^{-1}$ $MgSO_4$, and solidified with 2.5 $g \cdot L^{-1}$ Gelzan and supplemented with 22 μM TDZ and 2.9 μM gibberellic acid (GA_3). Filter sterilized GA_3 was added to cooled autoclave media before dispensing. Callus was incubated under 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white fluorescent light with a 16/8-h light/dark photoperiod for 4 weeks to determine embryogenic or organogenic potential.

The experiments were a 2 (auxin type) × 4 (auxin concentration) factorial design with 10 replicates including five subsamples (explants) for each cultivar (as separate experiments) arranged in a completely randomized design. Data were collected for the percentage of explants forming callus (see Fig. 6 later in the article) and regenerative callus (see Fig. 6B and C). Data sets were subjected to analysis of variance (ANOVA), and means were separated using Fisher's LSD. Trends were evaluated using regression analysis (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Callus induction—carbohydrate response. In the second experiment, we investigated the effects of carbohydrates sucrose, glucose, and fructose at 3%, 6%, or 9% concentrations on callus production. Basal medium for all experiments consisted of full-strength MS salts and vitamins, 0.1 $g \cdot L^{-1}$ MES, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.75 $g \cdot L^{-1}$ $MgSO_4$, and media was solidified with 2.5 $g \cdot L^{-1}$ Gelzan. Auxin type and concentration were based on the results from the previous experiment (i.e., 10 μM 2,4-D for Carefree Beauty™ and Italian Ice® and 5 μM 2,4,5-T for Ringo All-Star™). Media were adjusted to a pH of 5.75 before autoclaving. Actively growing leaflets were scored across the midrib and placed abaxial side down and cultured in the dark in a three (type) × four (concentration) factorial arrangement. Each experiment consisted of 10 replicates with five subsamples (explants) for each cultivar (as separate experiments) arranged in a completely randomized design. After 4 weeks, callus was transferred to a regeneration media consisting of half-strength MS salts with full-strength MS vitamins, 3% sucrose, 0.1 $g \cdot L^{-1}$ MES, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.75 $g \cdot L^{-1}$ $MgSO_4$, solidified with 2.5 $g \cdot L^{-1}$ Gelzan, and supplemented with 22 μM TDZ for an additional 4 weeks to determine regeneration potential. Data were collected for the percentage of explants producing callus and regenerative callus. Data were subjected to ANOVA and means were separated using Fisher's LSD. Trends were evaluated using regression analysis (Proc GLM, SAS Version 9.1).

Shoot regeneration—cytokinin response. To determine responses of shoot regeneration to cytokinins, callus was induced in the dark on media consisting of full-strength MS salts and vitamins, 0.1 $g \cdot L^{-1}$ MES, 0.1 $g \cdot L^{-1}$

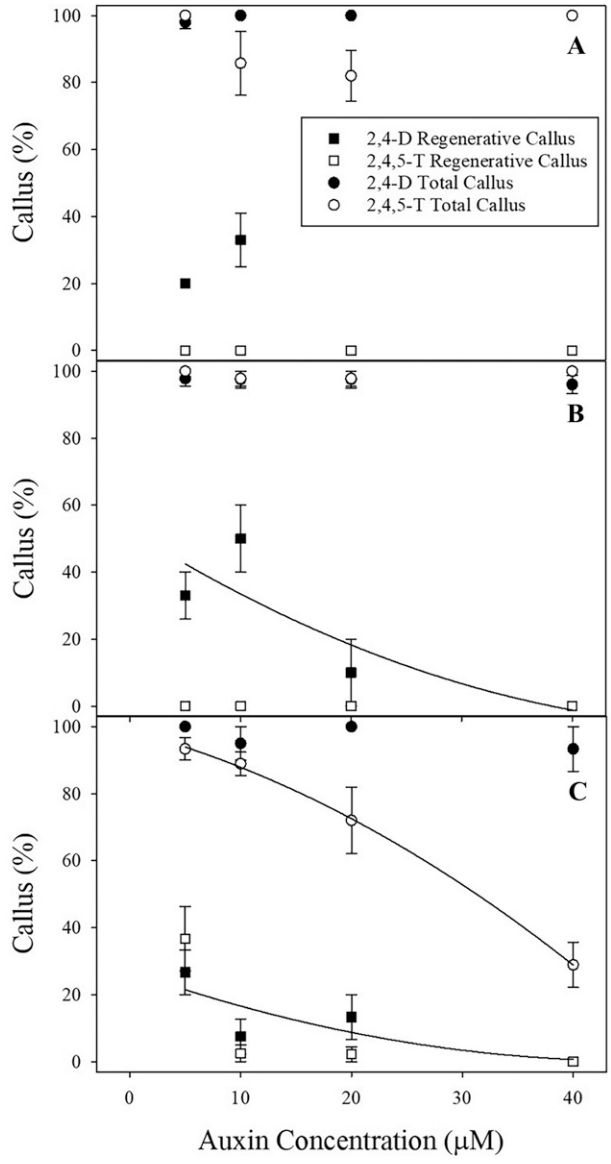

Fig. 1. Effect of auxins 2,4-D and 2,4,5-T on percentage of leaf explants producing total callus and regenerative callus for the three rose cultivars. (**A**) Italian Ice®, (**B**) Carefree Beauty™; 2,4-D regenerative callus: $y = 52.3 - 2.07x + 0.02x^2$, $r^2 = 0.85$; (**C**) Ringo All-Star™; 2,4,5-T total callus: $y = 98.9 - 0.89x - 0.02x^2$, $r^2 = 0.99$, 2,4-D regenerative callus: $y = 27.0 - 1.2x + 0.01x^2$, $r^2 = 0.81$. Symbols represent means ± standard error.

myo-inositol, 0.75 g·L^{-1} $MgSO_4$, solidified with 2.5 g·L^{-1} Gelzan, and supplemented with the optimal auxin and carbohydrate determined for each cultivar in previous experiments (i.e., 10 µM 2,4-D and 60 g·L^{-1} glucose for Carefree Beauty™, 10 µM 2,4-D and 30 g·L^{-1} sucrose for Italian Ice®, and 5 µM 2,4,5-T and 30 g·L^{-1} sucrose for Ringo All-Star™). After 4 weeks, explants were transferred to regeneration media consisting of half-strength MS salts and vitamins, 0.1 g·L^{-1} myo-inositol and MES, 2.9 µM GA_3, 0.75 g·L^{-1} $MgSO_4$, and 2.5 g·L^{-1} Gelzan and supplemented with either 5.0, 10, 20, or 40 µM TDZ or BA. Each experiment consisted of 10 replicates with five subsamples arranged in a completely randomized design. Cultures were incubated under 40 µmol·m^{-2}·s^{-1} cool-white fluorescent light with a 16/8-h light/dark photoperiod at a temperature of ~23 °C. Explants were incubated for 4 weeks. Data were collected on the percentage of callus that produced regenerative shoots and the number of shoots that formed per callus piece. Data were subjected to ANOVA and means were compared using Fisher's LSD. Trends were evaluated using regression analysis (Proc GLM, SAS Version 9.1).

Rooting—auxin response. Regenerated shoots were transferred to shoot multiplication/maintenance media as described earlier. After 4 weeks, shoots ~25 mm in length were transferred to 180-mL glass jars containing 25 mL of rooting media consisting of full-strength MS salts and vitamins, 30 g·L^{-1} sucrose, 0.1 g·L^{-1} myo-inositol, 0.1 g·L^{-1} MES, and 6.5 g·L^{-1} agar supplemented with 0, 1.25, 2.5, 5.0, or 10 µM IAA. Medium pH was adjusted to 5.8. After 4 weeks, shoots were transferred ex vitro and acclimatized in growing media (2 peat:1 vermiculite, v:v) in 50-cell trays and placed under intermittent mist (10-s duration at 10-min intervals).

Results and Discussion

Callus induction—auxin response. Callus induction was achieved in all three genotypes for all auxin treatments. For Italian Ice® and Ringo All-Star™, callus induction was significantly influenced by auxin source, concentration, and their interaction ($P < 0.05$; Fig. 1A and C). For Carefree Beauty™, callus induction was significantly influenced by the interaction of auxin source and concentration ($P < 0.05$; Fig. 1B). The different 2,4-D concentrations had no significant effect on total callus induction for all the three rose cultivars ($P > 0.05$) (Fig. 1). Similarly, 2,4,5-T concentrations did not significantly influence the number of explants producing callus for Italian Ice® (Fig. 1A) and Carefree Beauty™ (Fig. 1B). In Ringo All-Star™, however, there was a significant curvilinear decline in the number of explants producing callus in response to increasing 2,4,5-T concentrations (Fig. 1C).

After 4 weeks, callus was transferred to half-strength MS media supplemented with 22 µM TDZ and 2.9 µM GA_3 for 4 weeks to examine the percentage of regenerative callus (i.e., callus that can produce either somatic embryos or shoots via somatic embryogenesis or organogenesis). There was no regenerative callus for Italian Ice® explants treated with 2,4,5-T, but Italian Ice® explants treated with 10 µM 2,4-D exhibited significantly higher regenerative callus percentage than other treatments

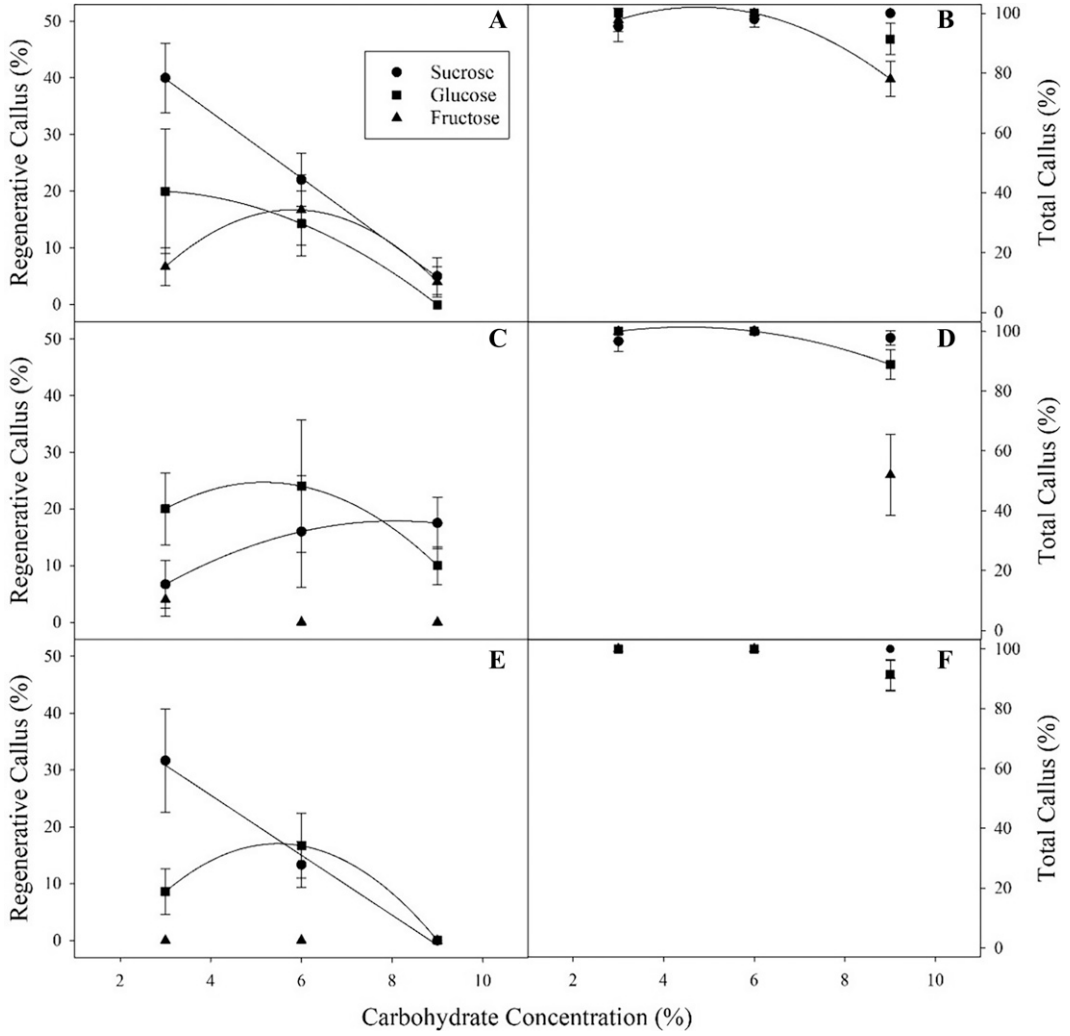

Fig. 2. Effect of carbohydrates, sucrose, glucose, and fructose on percentage of leaf explants producing callus and regenerative callus for the three rose cultivars. (**A**) Italian Ice®: sucrose y = 57.3 – 5.83x, r^2 = 0.99, glucose y = 17.1 + 2.4x – 0.48x^2, r^2 = 1.0, fructose y = –26.09 + 14.71x – 1.26x^2, r^2 = 1.0; (**B**) Italian Ice®: fructose total callus y = 71.3 + 12.85x – 1.35x^2, r^2 = 1.0; (**C**) Carefree Beauty™: sucrose y = –10.49 + 7.03x – 0.44x^2, r^2 = 1.0, glucose y = –2.0 + 10.3x – x^2, r^2 = 1.0; (**D**) Carefree Beauty™: glucose total callus y = 88.89 + 5.56x – 0.62x^2, r^2 = 1.0; and (**E and F**) Ringo All-Star™: sucrose y = 46.7 – 5.28x, r^2 = 0.99, glucose y = –24.3 + 15.09x – 1.38x^2, r^2 = 1.0. Open symbols represent total callus formation, and closed symbols represent regenerative callus.

($P < 0.05$, Fig. 1A). There was no regenerative callus for Carefree Beauty™ explants treated with 2,4,5-T, whereas the percentage of regenerative callus exhibited a quadratic decrease in response to increasing 2,4-D concentration (Fig. 1B). For Ringo All-Star™, there was significantly higher regenerative callus percentage for explants treated with 5 μM 2,4,5-T (37% ± 10%) and 5 μM 2,4-D (27% ± 7%), and the percentage of regenerative callus decreased quadratically with increasing 2,4-D concentration ($P > 0.05$, Fig. 1C).

The induction of regenerative callus from rose leaf explants in response to auxin treatments has been shown to be highly cultivar specific. Reports showed that 2,4-D concentrations ranging from 2.3 μM for 'Pariser Charme' and 'Heckenzauber' to 90.5 μM for 'Linda' have been suitable for induction of regenerative callus (Dohm et al., 2001; Zakizadeh et al., 2008). In the present study, 10 μM 2,4-D produced the highest percentage of regenerative callus for Italian Ice® and Carefree Beauty™. This is consistent with Hsia and Korban (1996), who found 10 μM 2,4-D produced the highest level of regenerative callus on Carefree Beauty™.

Interestingly, either 5 μM 2,4-D or 5 μM 2,4,5-T produced the highest levels of regenerative callus in Ringo All-Star™ (Fig. 1C). Whereas 2,4-D has been the predominant auxin used to induce regenerative callus in *Rosa* sp., 2,4,5-T has shown to be beneficial for inducing regenerative callus in some rose cultivars. For example, Estabrooks et al. (2007) found 2,4,5-T more effective than 2,4-D for inducing callus in cultivar Livin' Easy. Our study also shows that 2,4,5-T can initiate regenerative callus as well as 2,4-D in Ringo All-Star™, indicating the response should be tested for each cultivar individually.

Callus induction—carbohydrate response. Some callus induction was achieved in all three genotypes for all sucrose, glucose, and fructose treatments. Total callus production was consistently high across all concentrations of sucrose and glucose in all three cultivars (Fig. 2). For Italian Ice®, callus induction was influenced by carbohydrate concentration ($P < 0.05$; Fig. 2B). For Carefree Beauty™, callus induction was significantly influenced by carbohydrate source and concentration and by their interaction ($P < 0.05$; Fig. 2D). Specifically for Carefree Beauty™, total callus production was close to 100% for the glucose treatment before attenuating in a quadratic fashion at higher concentrations. Similarly, callus production was high in cultivars at low concentrations of fructose before significantly declining at higher concentrations in Italian Ice® and Carefree Beauty™ ($P < 0.05$; Fig. 2).

After 4 weeks, callus was transferred to half-strength MS media supplemented with 22 μM TDZ for 4 weeks to examine the percentage of regenerative callus (Fig. 2). For Italian Ice®, the percentage of regenerative callus was significantly influenced by both carbohydrate type, concentration, and their interaction ($P < 0.05$; Fig. 2A). The highest regeneration was obtained using 3% sucrose before declining in a linear fashion with increasing sucrose concentrations (Fig. 2A). Glucose and fructose treatments resulted in regenerative calli but at lower rates than sucrose.

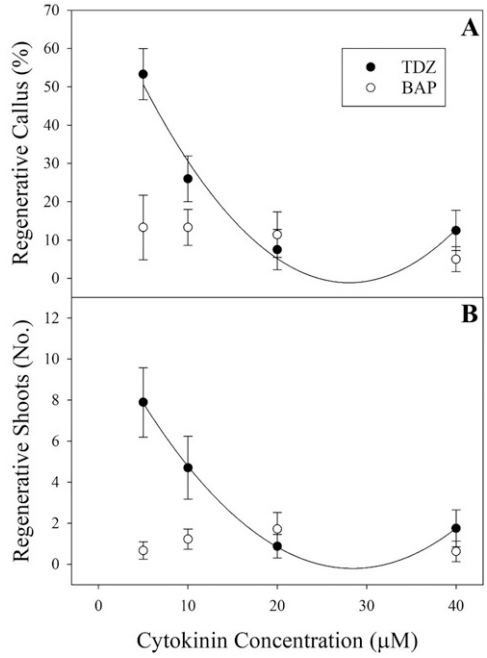

Fig. 3. The effect of thidiazuron (TDZ) and 6-benzylaminopurine (BAP) on the number of regenerative callus percentage ($y = 75.2 - 5.43x + 0.097x^2$, $r^2 = 0.97$; (**A**) and the number of regenerative shoots per callus ($y = 10.95 - 0.71x + 0.012x^2$, $r^2 = 0.99$; (**B**) for Italian Ice®. Symbols represent means ± standard error.

For Carefree Beauty™, the percentage of regenerative callus was significantly influenced by the concentration of both sucrose and glucose and their interactions, but not by fructose ($P < 0.05$; Fig. 2C). The regenerative callus percentage increased quadratically with increasing sucrose concentrations and exhibited a quadratic response to increasing glucose concentrations. (24% ± 11.7%).

Ringo All-Star™ showed a similar response to sucrose and glucose as Italian Ice® because the percentage of regenerative callus in both cultivars was significantly influenced by carbohydrate source, concentration, and

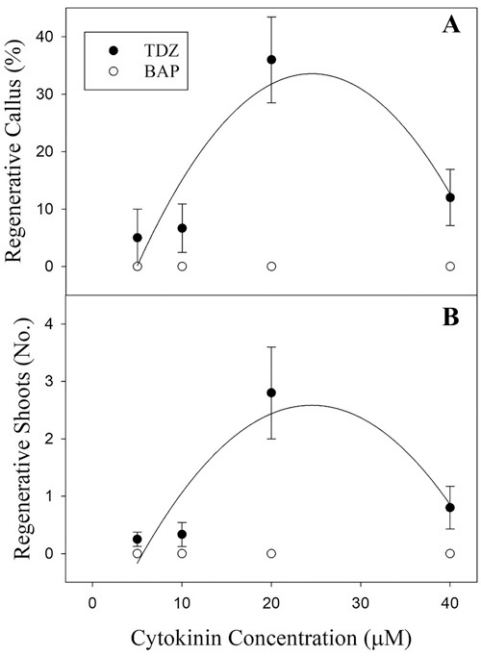

Fig. 4. The effect of thidiazuron (TDZ) and 6-benzylaminopurine (BAP) on regenerative callus percentage ($y = -19.1 + 4.30x - 0.088x^2$, $r^2 = 0.82$; (**A**) and the number of regenerative shoots per callus ($y = -1.77 + 0.36x - 0.007x^2$, $r^2 = 0.79$; (**B**) for Carefree Beauty™. Symbols represent means ± standard error.

their interaction ($P < 0.05$; Fig. 2E). The highest percentage of regenerative callus was obtained on sucrose, exhibiting a linear decline with increasing sucrose concentrations. Regenerative callus percentage exhibited a quadratic response in increasing glucose concentrations, and no regenerative callus was observed with fructose.

Sucrose is commonly used for the induction of regeneration-competent callus in rose. A 3% sucrose concentration is commonly used for rose regeneration (Kim et al., 2003, 2004; Kintzios et al., 1999; Marchant et al., 1996; Rout et al., 1991; Vergne et al., 2010). However, 2% sucrose has been reported as effective for 'Tineke', 'Domingo', and 'Selfluor' (de Wit et al., 1990), whereas the combination of 2% sucrose and 1% glucose has been used for 'Linda' (Zakizadeh et al., 2008). Interestingly, we found that regenerative callus formation occurred on media containing 6% glucose for Carefree Beauty™. Similarly, Hsia and Korban (1996) found the highest regenerative callus for Carefree Beauty™ was produced on media containing 6% glucose, and Bao et al. (2012) reported higher somatic embryo induction and less morphologically abnormal somatic embryos on 3% and 6% glucose than on sucrose for 'Samantha'. However, Burrell et al. (2006) reported greater embryogenic callus development on sucrose than glucose for Carefree Beauty™. Occasionally, alternative sugars such as maltose have been used with limited success in 'Frensham', 'Tineke', 'Dr. Huey', and 'Trumpeter', and *R. rugosa* (Castillon and Kamo, 2002; Kunitake et al., 1993; Yokoya et al., 1995).

Effect of cytokinin source and concentration on regenerative callus initiation and regeneration. The percentage of regenerative callus and the number of regenerative shoots per callus were influenced significantly by cytokinin type and concentration and their interaction for Italian Ice® (Fig. 3), Carefree Beauty™ (Fig. 4), and Ringo All-Star™ (Fig. 5). Both responses exhibited a quadratic trend in the three cultivars with increasing TDZ concentrations. The highest percentage of regenerative callus and the highest number of regenerative shoots per callus were achieved with media containing 5 µM TDZ for Italian Ice® (53.3 ± 6.67%; 7.9 ± 1.7), 20 µM TDZ for Carefree Beauty™ (36.0 ± 7.45%; 2.8 ± 0.8), and 5 µM TDZ Ringo All-Star™ (32.0 ± 6.97%; 2.88 ± 1.01). However, relatively low levels of regeneration and no regeneration occurred for Italian Ice® and Carefree Beauty™ when using BAP, respectively, and only regenerative shoots were observed for Ringo All-Star™ on 5 and 10 µM BAP.

TDZ is commonly used for regeneration of shoots from explants. Here, consistent with previous reports, we found variability with concentrations of TDZ between rose cultivars. For example, Li et al. (2002) and Hsia and Korban (1996) found that 2.3 and 23 µM of TDZ, respectively, were beneficial for Carefree Beauty™. Zakizadeh et al. (2008) reported that 45.4 µM of TDZ was successful for 'Etna', whereas TDZ concentrations between 4.5 and 45.4 µM were sufficient for 'Sonja' and 'Linda'. TDZ was also reported to enhance somatic embryo germination for 'Samantha' (Bao et al., 2012) and to improve regeneration in several unspecified rose cultivars (Ludwig et al., 2000).

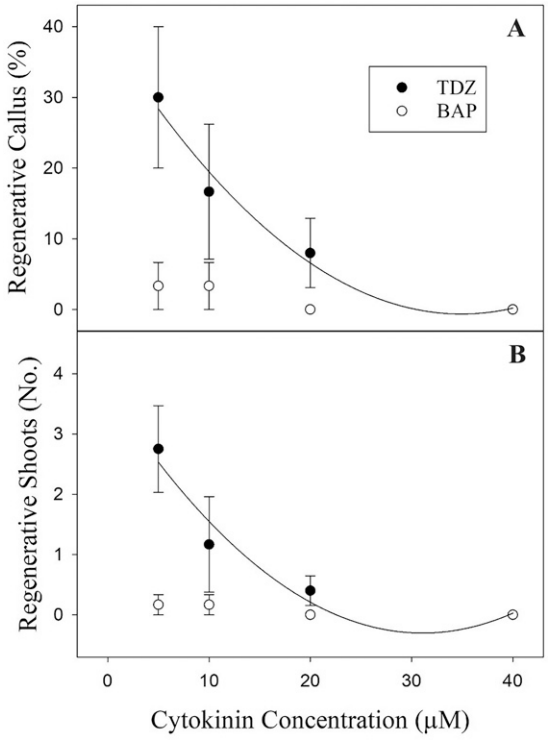

Fig. 5. The effect of thidiazuron (TDZ) and 6-benzylaminopurine (BAP) on regenerative callus percentage ($y = 41.0 - 2.18x - 0.029x^2$, $r^2 = 0.99$; (**A**) and the number of regenerative shoots per callus ($y = 3.88 - 0.25x + 0.004x^2$, $r^2 = 0.99$; (**B**) for Ringo All-Star™. Symbols represent means ± standard error.

Although BAP is commonly used for rose micropropagation, we found its effectiveness in inducing shoots from regenerative callus varies (Figs. 3–5). Li et al. (2002) found similar rates of regeneration on media containing BAP for Carefree Beauty™ compared with media containing TDZ. Rout et al. (1991) and Kim et al. (2003) used BAP at 2.2 µM for 'Landora' and 4.4 µM for 'Sumpath', respectively, to promote regeneration. However, BAP was found to be ineffective in promoting regeneration for 'Tournament of Roses', 'Fourth of July', 'Graham Thomas', and 'Sequoia Ruby' (Kim et al., 2004). Also, BAP in combination with GA_3 and NAA did not induce regeneration for 'Baccara', 'Mercedes', 'Ronto', or 'Soraya' (Kintzios et al., 1999).

For all cultivars, shoots derived from callus were successfully propagated using standard rose maintenance media (Fig. 6). Microcuttings were successfully rooted on media containing 2.5, 5.0, or 10 µM IAA and transferred ex vitro. Shoots that were not treated with auxin did not survive when transferred ex vitro.

In conclusion, this study provides regeneration protocols for the highly valued rose Carefree Beauty™ and the two newer cultivars Italian Ice® and Ringo All-Star™. The variability in response among the three cultivars is consistent with what has previously been reported in the literature, suggesting regeneration protocols need to be refined for individual cultivars. The results presented here will provide a strong platform for the development of transformation and genome editing for these elite rose cultivars.

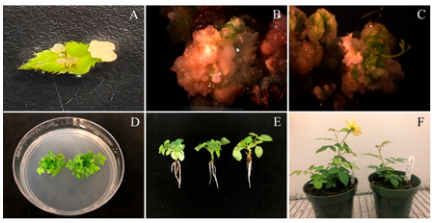

Fig. 6. Regeneration of Italian Ice®. (**A**) Embryogenic callus induced from in vitro leaf explants. (**B**) Early-stage regenerating callus masses. (**C**) Group of regenerating somatic embryos/meristems. (**D**) Regenerated shoots cultured on rose maintenance media. (**E**) Rooted plantlets. (**F**) Regenerated plants.

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