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Shoot regeneration from leaf segments of peach × almond hybrid, GF677 rootstock

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Abstract. The GF677, a peach × almond hybrid, is a promising rootstock for both peaches and almonds and is exploited worldwide. To improve this rootstock via genetic engineering is prerequisite to have an efficient regeneration protocol. In order to evaluate the effect of different concentrations of auxin and cytokinin upon shoot regeneration from leaf segments of GF677 plantlets grown in vitroin AP medium containing 0.5 mg/l BAP, 4 mg/l GA_3, and 200 mg/l casein hydrolysate was used. Leaf segments were placed with abaxial side down on regeneration media with NAA (0, 0.2, and 0.3 mg/l) and BAP (0.5, 1, 1.5, and 2 mg/l). Callus induction was observed in all treatments, but it was not influenced by different combinations of plant growth regulators. Substitution of BAP with TDZ evoked similar results in terms of callus induction and callus growth with no shoot regeneration. Among the three cytokinins investigated (TDZ, BAP, and Zeatin), only TDZ caused shoot induction in GF677 leaf segments on AP medium. Finally, to determine the most efficient TDZ level, shoot induction was recorded in all evaluated TDZ concentrations (5, 10, and 15 mg/l) without any significant difference among them.
Key Words: GF677, leaf segment, shoot regeneration, TDZ.

Introduction. GF677, a peach × almond hybrid, exploited as a suitable and compatible rootstock for peach and almond, have high tolerance to calcareous, dry and less fertile soils (Ehsanpour & Amini 2001). Biotechnological approaches are a matter of choice to broaden the gene pool and achieve better genotypes. However, developing an efficient method for shoot regeneration from a mature tissue (mostly leaf) appears to be a prerequisite for genetic engineering of any plant, including GF677 (Ainsley et al 2001; Litz & Gray 1992).

Growth regulators are the most important influence factors in shoot regeneration (Bhojwani & Razdan 1996). In *Prunus* spp., some growth regulators such as benzyl-amino-purine (BAP), kinetin (Kin), zeatin and 6-(gamma, gamma-dimethylallylamino) purine (2ip) have been exploited for shoot regeneration; nevertheless, the number of the shoots per explant reported is fairly low (Mant et al 1989). In some experiments, thidiazuron (TDZ) has been more efficient compared to BAP (Bhagwat & Lane 2004; Perez-Tornero et al 2000; Staniene & Stanys 2004). Moreover, TDZ has been used for shoot regeneration from leaf segments of cherry (Hammatt & Grant 1998). However, BAP was more efficient in shoot formation from leaves of cherry, sour cherry and peach (Gentil et al 2002; Tang et al 2002). In other *Prunus* species, BAP has been utilized for shoot regeneration from *in vitro* leaf explants. All of these results show that the efficiency of organogenesis greatly depends on the plant species, genotype, explant and the growth regulators (Antonelli & Druat 1990).

In almond, BAP and TDZ have been successfully used to induce adventitious shoots (Ainsley et al 2000). Shoot regeneration from *in vitro* cultured leaves of apricot was achieved by using TDZ and naphthalene-acetic acid (NAA) (Perez-Tornero & Burgo 2004). Adventitious shoot formation is being significantly affected by the type and concentration of the auxin used in regeneration media. Accordingly, indol-butyric acid (IBA) and NAA could improve adventitious bud development in almond (Ainsley et al 2001). In another

experiment of cherry shoot regeneration on leaf segments was obtained in the media with NAA and TDZ (Bhagwat & Lane 2004).

There is no information based on our knowledge about shoot regeneration in GF677 and this is the first report of shoot regeneration from leaf segments of this valuable rootstock.

Material and Method. The leaves were dissected from *in vitro* plantlets grown in the AP basal medium (Almehdi & Parfitt 1986) supplemented with BAP 0.5 mg/L and GA_3 0.5 mg/L for about 3 months (Figure 1) and cut into 1 to 0.5 cm segments followed by transferring their abaxial surface on MS medium supplemented with NAA (0, 0.2, and 0.4 mg/L) along with BAP (first experiment) or TDZ (second experiment), each 0.5, 1, 1.5, and 2 mg/l regeneration media. The cultures were kept in darkness at 24±2°C for one month followed by incubation of one month in light condition at 3000-4000 lux provided by white fluorescent lamps. Percent of callus formation, number of leaves turned yellow and brown, in darkness as well, as percent of callus formation and callus color in light were measured in these two experiments.

Figure 1. GF 677 plantlets *in vitro* used for shoot regeneration.

The calli were transferred into AP medium supplemented with the above mentioned growth regulators with the same concentrations and with BAP 5 mg/L and IBA 0.1 mg/L.

In the third experiment, the leaves from the in vitro grown plantlets were cut into 0.5 –1 cm long segments and put onto the AP medium having a combination of TDZ, zeatin and BAP with the abaxial side down (Table 1). Then all were incubated at 24±4ºC in darkness for one month and after that in lightness at 3000-4000 lux intensity and the same traits were measured. Hormones were sterilized through filtering with Millipore and autoclaved before added to the media.

The regenerated plants from T3 and T4 were transferred to the media without hormones for shoot elongation and those from the other treatments were transferred to the same regeneration media.

Table 1

Different plant growth regulators combination used in the third experiment

T1	*T2*	*T3*	*T4*	*T5*	*T6*	*T7*
0.1 IBA+5 BAP	0.1 IBA+10 BAP	5 TDZ	10 TDZ	5 Zeatin	5 BAP+5 TDZ	5 BAP+5 Zeatin

Furthermore, to find the proper TDZ concentration for *in vitro* shoot regeneration, leaf explants were laid on AP medium containing 5, 10 and 15 mg/L of TDZ (forth experiment) and maintained in the same conditions.

Afterwards, regenerated explants were passed into shoot elongation medium (without PGR) and subsequently in the maintenance medium (AP medium containing BAP 0.5 mg/L and GA3 4 mg/L). Finally, well grown shoot were exploited for rooting step in AP medium supplemented with IBA 2 mg/L.

In all experiments, we used completely randomized design with 8-10 replications (Petri dishes). In each Petri dish, were 5-10 leaf explants.

Analysis of variance was performed using SPSS program through a factorial approach and Duncan multiple range test were utilized for mean comparisons at $P<0.05$.

Results and Discussion. In the first experiment, callus induction was observed in all media containing BAP and NAA (Figures 2 and 3), in two nodulous and non-nodulous forms (Figure 4). Callus was inducted only in the BAP presence, illustrating that callus induction from the GF677 leaf explants can occur in the media containing cytokinin (even low concentrations of BAP) (Table 2).

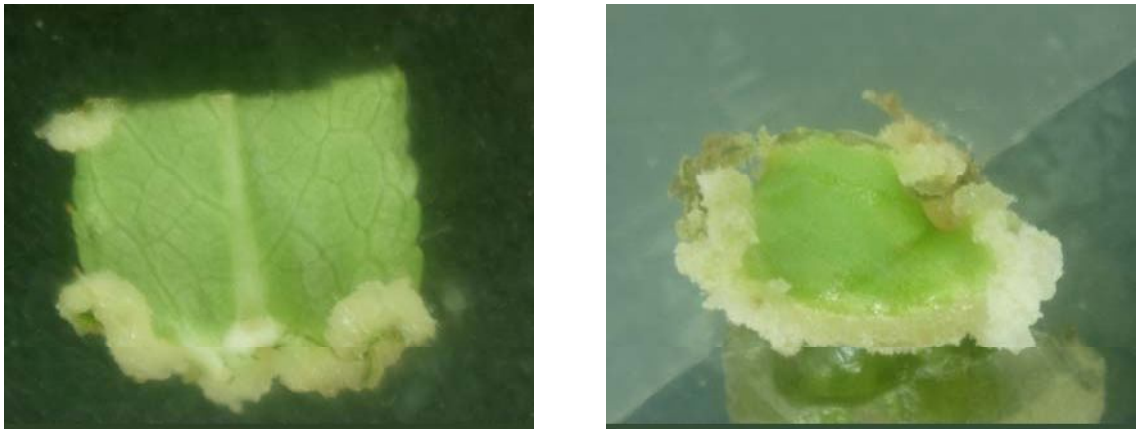

Figure 2. Callus initiation on callus induction medium in darkness.

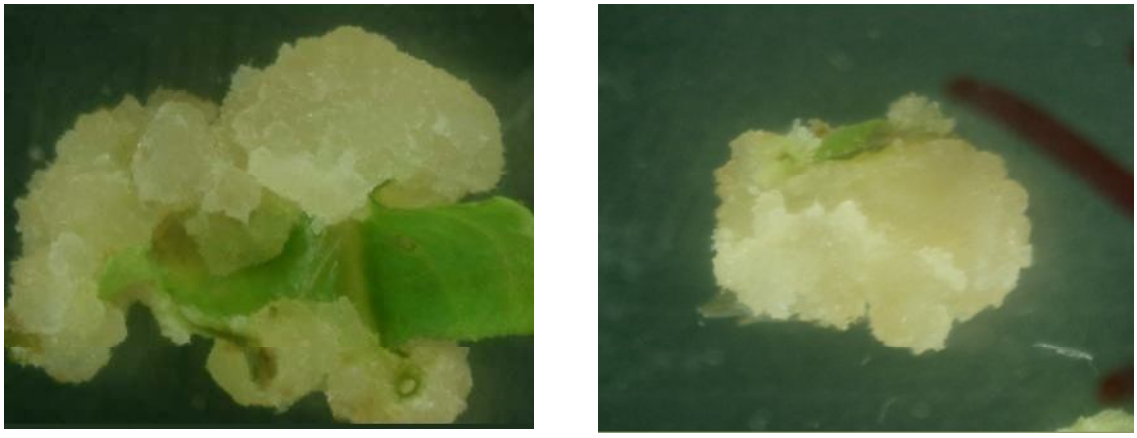

Figure 3. Non-nodular callus growth in light.

Effect of BAP and NAA was not significant on callus induction percentage; so that callus induction in the medium without NAA was the same as in the NAA containing medium. From the other point of view as it is apparent in Table 2, different concentrations of BAP had similar effects on callus induction in darkness and light conditions and it was concluded that BAP as low as 0.5 mg/L was efficient in callus induction from leaf segments. The interaction between BAP and NAA upon percentage of callus induction was

significant in darkness and not in light. Therefore, callus induction was influenced by the effects of these two growth regulators.

Table 2

The mean callus induction in darkness and light on BAP

Traits		*BAP concentration (mg/l)*
Callus induction at light (%)	*Callus induction at darkness (%)*	
87.63±18.53 a	71.66±26.10 a	0.5
85.46±20.50 a	73.86±21.65 a	1.0
85.53±23.36 a	78.83±24.71 a	1.5
83.99±17.61 a	66.10±24.39 a	2.0

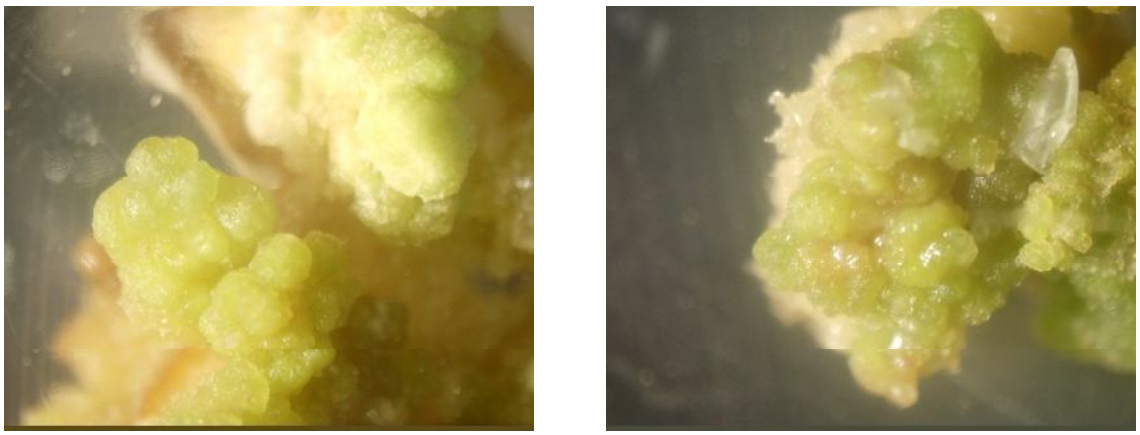

Figure 4. Nodular callus growth in light.

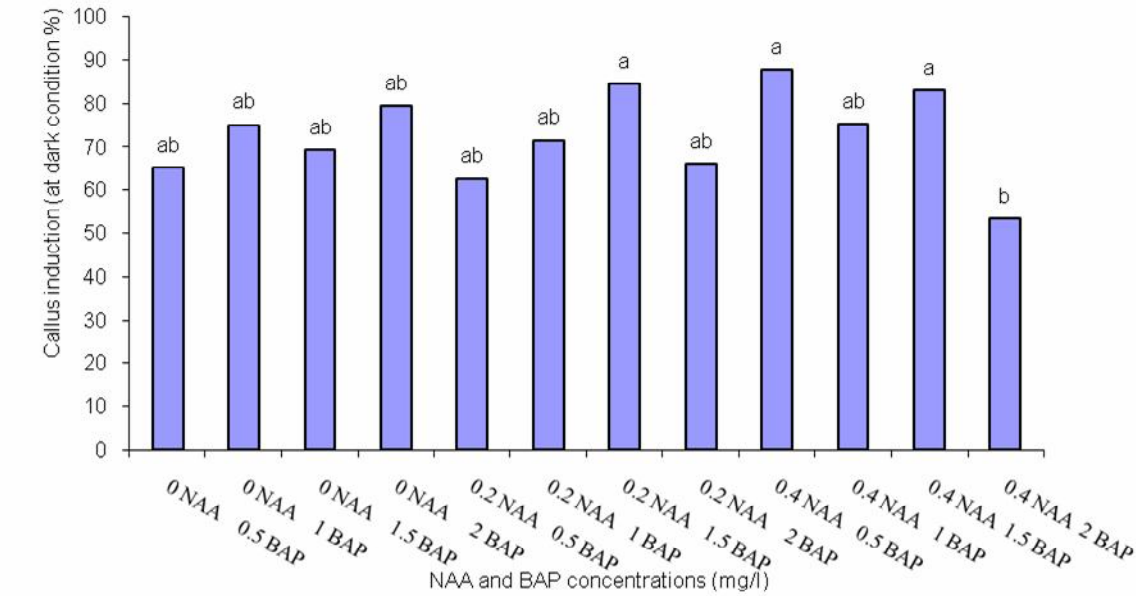

Figure 5. Callus induction percentage in darkness at different BAP and NAA concentration.

The highest rate of callus induction was observed when 1.5 mg/L BAP and 0.2 mg/L NAA was used and the lowest rate occurred with 2 mg/L BAP and 0.4 mg/L NAA (Figure 5).

Shoot regeneration is highly dependent on genotype and the type of cytokinin. There are controversial results about the efficiency of BAP on regeneration from leaf segments in almond. Miguel et al. (1996) showed that the almond cultivar Boacosta, has a negative response to BAP. In another study, it was shown that the formation of adventitious buds from leaves was affected by the cytokinin's type and concentration, also the concentration of NAA and that BAP was less effective than the other cytokinins in shoot induction (Pereze-Tornero & Burgo 2004).

Accordingly, our results showed that there was no shoot induction in the first subculture, illustrating the insufficiency of BAP and NAA in shoot induction from leaf segments. Subcultures to the media with and without BAP also carried out but without any result. In contrary, BAP has been used for inducing adventitious shoots in two almond cultivars (Ainaley et al 2001) as well as other *Prunus* species (Antonelli & Druat 1990).

In the second experiment, callus induction occurred in all culture media containing TDZ and NAA and showed that callus induction from leaf segments of GF677 could take place in the presence of TDZ even in the lack of auxin. Therefore, it seems that the internal auxin amount is high enough to induce callus formation.

Different concentrations of NAA had significant effects on callus induction being 74% in the absence of NAA and 82% when 0.4 mg/L NAA was used; however, the other recorded traits were not affected (Table 3). On the other hand, the lowest amount of callus induction was observed when 0.5 mg/L TDZ was used and the highest at 1.0 mg/L TDZ (Table 4). Similar results were seen when TDZ and NAA were used together. The lowest callus induction was at 0.5 mg/L TDZ with any concentration of NAA in darkness and with 0.2 mg/L NAA in the light. Nevertheless, the highest callus induction was recorded at 1.0 mg/L TDZ with 0.2 and 0.4 mg/L NAA as well as at 1.5 mg/L TDZ without NAA in darkness and 1.0 mg/L TDZ with 0.4 mg/L NAA in the light (Table 5). Subculturing of the calli on the same media as well as on the higher concentrations of TDZ was carried out, but no shoot induction was occurred.

Table 3

The mean callus induction in darkness and light on NAA

Traits		*NAA (mg/l)*
Callus induction at light (%)	*Callus induction at darkness (%)*	
74.04±30.03 b	55.28±32.66 a	0.0
64.28±36.44 b	50.76±37.89 a	0.2
82.96±20.47 a	56.87± 31.48 a	0.4

Table 4

The mean callus induction in darkness and light on TDZ

Traits		*TDZ (mg/l)*
Callus induction at light (%)	*Callus induction at darkness (%)*	
55.00±42.42 c	26.59±4.04 b	0.5
87.64±16.63 a	70.23±1.23 a	1.0
72.22±27.72 b	59.37± 1.02 a	1.5
80.16±21.62 ab	61.01±0.3 a	2.0

The results of the first and second experiment revealed that the internal auxin of the leaf explants was high and so we used very low amounts of auxin in the third experiment. In all latter treatments callus induction from the leaf explants was observed in darkness and increased when were transferred to light. Vascular nodules were the witness in some treatments. Shoot induction was observed at 5.0 and 10.0 mg/L TDZ (with and without

auxin) which showed that shoot induction is possible from the leaf segments of GF677 using certain media and conditions (Figures 6-8).

Table 5

The mean callus induction in darkness and light on TDZ and NAA

Traits		*NAA and TDZ concentration (mg/l)*	
Callus induction at light (%)	*Callus induction at darkness (%)*	*NAA*	*TDZ*
55.00±44.72 b	29.16±29.22 bc	0.0	0.5
78.57±17.2 ab	60.71±34.93 ab	0.0	1.0
75.00±29.88 ab	75.00±26.72 a	0.0	1.5
87.5± 26.72 a	56.25±29.12 ab	0.0	2
18.75±29.12 c	6.25± 11.57 c	0.2	0.5
90.62±12.93 a	75.00±29.88 a	0.2	1.0
66.66±34.15 ab	56.25±34.71 ab	0.2	1.5
81.11±16.54 ab	65.55±30.76 a	0.2	2
91.25±12.17 a	44.37±32.78 ab	0.4	0.5
93.75±17.67 a	75.00±26.72 a	0.4	1.0
75.00±23.14 ab	16.87±36.44 ab	0.4	1.5
71.87±20.86 ab	61.25±24.16 ab	0.4	2

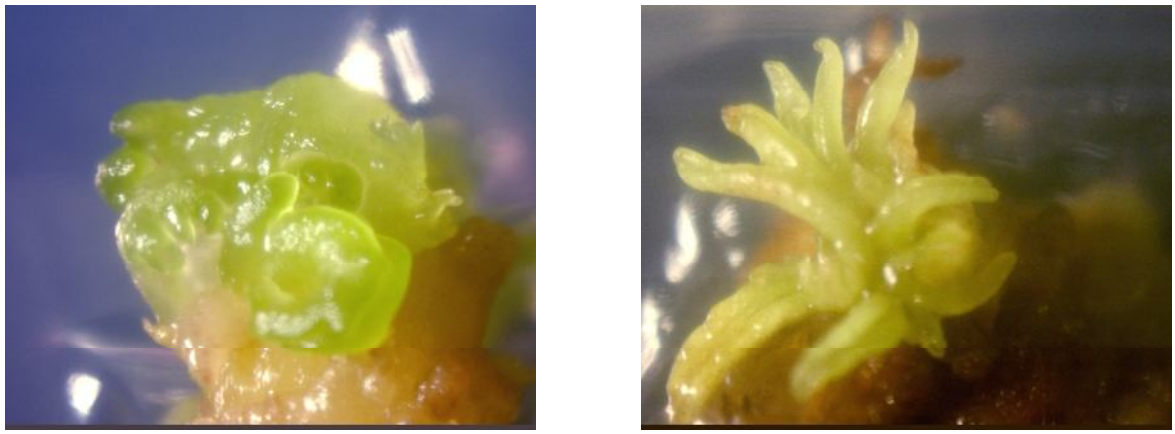

Figure 6. Shoots induction and formation on TDZ containing medium.

Figure 7. Shoot growth on TDZ containing medium.

Figure 8. Shoot growth on hormone free medium.

It was shown that different combinations of plant growth regulators had significant effects upon callus induction in both, darkness and light, upon percent of nodular callus in light and callus diameter in darkness. The highest amount of callus induction in darkness and light and callus diameter in darkness was on the medium containing 5.0 mg/L BAP. Furthermore, on the medium having 10.0 mg/L BAP the lowest amount of callus induction and growth was occurred. All cytokinin treatments had acceptable amount of callus induction; however, 5.0 mg/L zeatin along with 5.0 mg/L BAP was found more efficient than zeatin alone and TDZ (5.0 and 10.0 mg/L) in callus induction and growth (Table 6).

In the third experiment, shoot induction was observed only at 5.0 and 10.0 mg/L TDZ in 4 of 8 Petri dishes; however, shoot induction rate was 10 and 20 percent, respectively.

Mostly, there was one shoot per each callus, but in some cases, up to three shoots were observed on one callus. Because of shoot induction absence at the other treatments, analysis of variance was not performed. The emerged shoots after growing on hormone free medium was transferred onto maintenance medium to allow further shoots growth (Figure 9). Afterward, the 2 cm long shoots were transferred onto rooting medium.

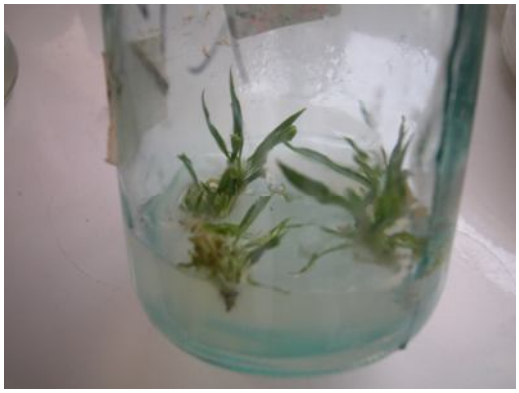

Figure 9. Further growth of produced shoots.

Mant et al (1989) succeeded to obtain shoots from three species of *Prunus* using BAP, Kin, zeatin, and 2ip while the number of shoots per explant has been reported to be a few. TDZ compared to BAP and other cytokinins has been proved to be more appropriate in shoot induction because of its high biological activity in most of plant species (Bhawat & Lane 2004; Staniene & Stanys 2004) and it was expected that shoot induction would be resulted on the lower amounts of it from GF677 leaf explants; nevertheless, this genotype seems recalcitrant to shoot induction and would need higher concentrations of TDZ.

Table 6

The means of callus induction, nodular callus and callus diameter in the combinations of TDZ, BAP, zeatin and IBA

Traits					*Hormonal treatments*			
In light			*In darkness*					
Callus diameter (mm)	*Nodal Callus (%)*	*Callus induction (%)*	*Callus diameter (mm)*	*Callus induction (%)*	*Z*	*IBA*	*BAP*	*TDZ*
8± 1.78 bc	0.0 b	51.50±16.9b	5.73±1.03c	35.5±22.94 c	-	0.1	10	-
10.13±1.19 a	0.0 b	97.5±5.0 a	9.42±0.93a	97.50± 5.0 a	-	0.1	5	-
6.8±1.34 c	49.09±9.7 a	87.62±14.43ab	6.5±1.35bc	75.25±3.5 ab	5	-	-	-
8.58±1.15 abc	0.0 b	76.00±33.61ab	7.69±0.59a	64.90±3.2 b	-	-	5	5
9.38±0.3 ab	0.0 b	93.4±21.97a	8.31±1.04a	90.18±12.2 ab	5	-	5	-
9.81±1.3 ab	40.02±18.2 a	90.36±13.8a	9.16±1.26a	84.24±15.2 ab	-	-	-	5
9.05±1.31 ab	39.84±28.8 a	93.93±7.42 a	8.37±1.52a	79.36±8.07 ab	-	-	-	10

Z - Zeatin.

A higher requirement of TDZ has also been reported in almond (Ainsley et al 2001). Ainsley et al (2001) could regenerate shoots from almond leaf segments using 5.0 mg/L TDZ along with 2.0 mg/L IBA, this is while in the present research no auxin was used with TDZ. It can be interpreted that the amount of internal auxin has been enough to make unnecessary any external auxin usage. To prove this hypothesis, we carried out the fourth experiment with TDZ and IBA. In the other *Prunus* species, it has been shown that low concentration of TDZ can cause shoot induction. Pereze-Tornero & Burgo (2004) used 0.5 and 2.0 mg/L TDZ (with NAA) for shoot induction from apricot.

In the fourth experiment, in all treatments, callus induction, growth and nodule formation were observed in darkness and light. The highest callus induction and percent nodular callus were occurred at 5.0 mg/L TDZ along with 0.01 mg/L IBA upon which the highest callus diameter was also detected in darkness. The highest callus diameter in the light was observed at 15.0 mg/L TDZ (Table 7).

For unknown reasons, there was no shoot induction observed in the media containing 10.0 mg/L TDZ while it had been occurred in the third experiment. In all other treatments apart of the fourth experiment, shoot induction was detected and shoots were transferred onto maintenance medium following their growth on hormone free medium. Afterward, shoots were transferred onto the rooting medium.

Analysis of variance revealed that there was no significant difference among the treatment in shoot induction and therefore, the concentration of 5.0 mg/L TDZ alone and in combination with 0.01 mg/L IBA at 15%, shoot induction were found to be the first-rate treatment for shoot induction from leaf segments of GF677 (Table 7).

Conclusions. In conclusion this paper describes a procedure for shoot induction of GF677 via callus regeneration and a successful growth of produced shoots. To the best of our knowledge this is the first report on the shoot induction from leaf explants. The present study showed that inducing high frequency organogenesis in leaf segments derived calli is possible by using 5.0 mg/L TDZ alone and in combination with 0.01 mg/L IBA. This efficient plant regeneration protocol can play an important role in generating transgenic plants from this multipurpose rootstock.

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