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RESEARCH ARTICLE

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Adventitious shoot regeneration from the leaves of *in vitro* grown 'Zhongli 1' pear (*Pyrus* spp.)

Abstract The pear (*Pyrus* spp.) is one of the most important temperate fruit crops. The technique of adventitious shoot regeneration from leaves is considered to be one of the shortcuts in the research on pear genetic modification and cellular engineering, which, however, has not been widely used. As the regeneration frequency of pear leaves is usually very low, the research on adventitious shoot regeneration from pear leaves is eagerly needed. In this experiment, the factors affecting shoot and bud regeneration from the leaves of 'Zhongli 1' pear were studied, and an efficient protocol for shoot regeneration was established. The results showed that different types of basic media, different combinations of plant growth regulators, leaf placement on medium, periods of dark culture and the use of silver nitrate ($AgNO_3$) on culture media all significantly affected the adventitious shoot regeneration frequency of 'Zhongli 1' pear. The details are as follows: (1) Among three kinds of basic media, NN69 was better for 'Zhongli 1' shoot regeneration, followed by half (½) MS, while full MS had no effect on shoot regeneration; (2) Thidiazuron (TDZ) was better than 6-benzylaminopurine (6-BA) for 'Zhongli 1' regeneration, with an optimal concentration of 1.5 $mg \cdot L^{-1}$, and the regeneration rate under this concentration could reach 85%, with 2.72 buds per leaf. 0.5 $mg \cdot L^{-1}$ indole-3-butyric acid (IBA), which induced a higher regeneration frequency, was a better choice for pear regeneration compared with 0.3 $mg \cdot L^{-1}$ naphthaleneacetic acid (NAA). Among the different combinations of plant growth regulators, TDZ + IBA was better for inducing high regeneration frequency; (3) The abaxial surface of leaves touching the medium was beneficial for leaves to uptake nutrients from the medium, and because of that, the regeneration frequency of leaves was significantly higher than that of leaves touching the medium with their adaxial surfaces (obverse side of leaf); (4) Dark culture was necessary for bud regeneration, and the best duration for dark culture of 'Zhongli 1' pear was 21 days; (5) The addition of 1.0 $mg \cdot L^{-1}$ $AgNO_3$ into the culture medium could promote adventitious shoot regeneration significantly. A high adventitious shoot regeneration frequency was obtained in this research, which will be beneficial for further research on efficient and stable *in vitro* plant regeneration systems and genetic modification of pear.

Keywords 'Zhongli 1' pear, adventitious shoot regeneration, growth regulators, tissue culture, *in vitro*

1 Introduction

Pear is one of the oldest and the most important temperate fruit crops. It belongs to the Rosaceae family and the Pomoideae sub-family, and is widely grown in temperate and sub-tropical regions of the world (Poudyal, 2007). The history of pear growing in China spans over 4000 years, and China is one of the centers of origin of many pear species like, *Pyrus bretschneideri*, *P. pyrifolia*, *P. ussuriensis*, etc. (Poudyal et al., 2008).

Adventitious shoot regeneration from leaves has been widely used in research on genetic and cellular engineering, especially in plant species improvement (Sun et al., 2004). However, an efficient *in vitro* plant regeneration system must be in place before such a technique can be employed. Up to now, many plants such as apple, banana

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and orange have obtained high adventitious shoot regeneration frequencies (Jun et al., 1993). Nonetheless, most fruit trees of perennial woody plants like the pear are always difficult in terms of regenerating buds from leaves. The regeneration frequencies from pear leaves are usually very low (Lane et al., 1998; Caboni et al., 1999). Although some European pears have achieved a higher regeneration rate (Leblay et al., 1991), it is still very difficult to get enough adventitious shoots from the leaves of Chinese and Japanese pears. There are already many researches on the adventitious shoot regeneration system of Chinese and Japanese pears, but few of them have reached satisfactory results. Therefore, studies on the efficient regeneration system of pears are necessary. The objective of this study was to study the factors affecting shoot regeneration and to establish an efficient protocol for pear regeneration and then to provide some references for pear vegetative propagation and genetic transformation.

2 Materials and methods

2.1 Plant materials

In vitro growing 'Zhongli 1' pear was used in this experiment. 'Zhongli 1', as a southern early maturing pear cultivar widely planted in China, is also called 'Lübaoshi' in Chinese, which was developed in 1982 from the cross between Shinseiki (*Pyrus pyrifolia* Nakai.)×Zaosu (*P. bretschneideri* Rehd.). This cultivar was bred by Zhengzhou Fruit Research Institution, China (Li et al., 2006).

2.2 Medium preparation and sterilization

The medium used for this experiment contained 6 $g \cdot L^{-1}$ agar and 30 $g \cdot L^{-1}$ sucrose, and the pH was adjusted to 5.8—6.0 with 1 $mol \cdot L^{-1}$ NaOH or 1 $mol \cdot L^{-1}$ HCl before the addition of agar and sucrose. The medium was sterilized at 121°C and at 0.12 kPa of pressure for 20 min. All other glassware and utensils were sterilized in an autoclave at 121°C and 0.12 kPa for 40 min, and all experiments were carried out under sterile conditions. Hormones were added in the medium before autoclave sterilization, and $AgNO_3$ used for the experiment was filtered and sterilized.

2.3 Leaf inoculation and culture conditions

In our experiment, tender leaves were excised from 20-day-old subcultured plantlets of *in vitro* growing 'Zhongli 1' pear and were subcultured on the proliferation medium (PM) of MS (Murashige and Skoog, 1962) + 1.0 $mg \cdot L^{-1}$ BA+ 0.1 $mg \cdot L^{-1}$ IBA (Table 1). Leaf petioles were removed, and three horizontal cuts along the midribs of the leaves were made and then the leaves were inoculated on different regeneration media (RM), with the abaxial surface (leaf back) touching the medium (The experiment on the effects of styles of leaf placement on the medium on adventitious shoot regeneration does not belong to this study). At the beginning of the culture period, the leaves were kept in continuous darkness for 21 d (The experiment on the effects of the dark culture periods on adventitious shoot regeneration does not belong to this study), and then they were transferred under a 16/8 light/dark photoperiod regime. After the shoots regenerated from the leaves, they were transferred to the shoot elongation medium (SEM) containing 0.5 $mg \cdot L^{-1}$ BA, 0.1 $mg \cdot L^{-1}$ IBA and 0.3 $mg \cdot L^{-1}$ gibberellic acid (GA_3) (Table 1). Cool white fluorescent tubes at the culture flask surface provided light at an intensity of 3000 lx. All cultures were maintained at (25 ± 2)°C.

2.4 Experimental design

Each bottle inoculated with 5 leaves, 4 bottles were considered as one replication, and each treatment was replicated 3 times, thus, in total, 60 leaves were used in one treatment. The number of shoots per leaf was recorded at the end of 60 d, and two indexes were calculated using the following equations:

(i) regeneration frequency = leaf number with adventitious shoots / total number of leaves used in the treatment × 100%;

Table 1 Culture media and their compositions used during shoot proliferation and adventitious shoot regeneration

item	PM/$mg \cdot L^{-1}$	RM/$mg \cdot L^{-1}$	SEM/$mg \cdot L^{-1}$
basic medium	MS	MS, 1/2MS and NN69	MS
growth regulators			
TDZ	–	0.5, 1.0, 1.5 and 2.0	–
BA	1	3.0, 4.0, 5.0 and 6.0	0.5
IBA	0.1	0.5	0.1
NAA	–	0.3	–
GA_3	–	–	0.3
agar	6000	6000	6000
sucrose	30000	30000	30000
$AgNO_3$	–	0 and 1.0	–

Note: PM = Proliferation medium; RM = Regeneration medium; SEM = Shoot elongation medium.

(ii) bud number per leaf = total number of adventitious shoots/total number of leaves used in the treatment $\times 100\%$.

2.5 Effects of basic media on adventitious shoots regeneration

Three different kinds of basic media were used in this experiment: MS, 1/2MS (MS nutrient elements were halved, with the quantity of agar and sucrose the same as in MS), and NN69 (Nitsch and Nitsch, 1969). Each basic medium had two levels of hormone concentrations: 1.0 $mg \cdot L^{-1}$ TDZ + 0.5 $mg \cdot L^{-1}$ IBA, and 2.0 $mg \cdot L^{-1}$ TDZ + 0.5 $mg \cdot L^{-1}$ IBA.

2.6 Effects of combinations of plant growth regulators on adventitious shoot regeneration

NN69 was used as a basic medium for this experiment with four different kinds of plant growth regulators: two cytokinins of TDZ and 6-benzylaminopurine (BA), and two auxins of IBA and naphthaleneacetic acid (NAA). Their concentrations were from 3 $mg \cdot L^{-1}$ to 6 $mg \cdot L^{-1}$ (BA), from 0.5 $mg \cdot L^{-1}$ to 2.0 $mg \cdot L^{-1}$ (TDZ), 0.5 $mg \cdot L^{-1}$ (IBA), and 0.3 $mg \cdot L^{-1}$ (NAA), respectively. The combinations of these four types of plant growth regulators are shown in Table 3.

2.7 Effects of leaf placement on the medium on adventitious shoot regeneration

The medium was NN69 + 1.0 $mg \cdot L^{-1}$ TDZ + 0.5 $mg \cdot L^{-1}$ IBA. Two different leaf placement styles were used in the experiment, one being the abaxial surface (leaf back) of leaves touching the medium surface, and the other the adaxial surface (obverse side of leaf) of leaves touching the medium.

2.8 Effects of the dark culture periods on adventitious shoot regeneration

The medium and hormonal concentration used in this experiment were NN69 + 1.0 $mg \cdot L^{-1}$ TDZ + 0.5 $mg \cdot L^{-1}$ IBA. Six different treatments were used in this experiment, i.e. the leaves separately kept in the dark for 7, 14, 21, 28 and 42 d, and then transferred under the 16/8 light/dark photoperiod regime. The control, without treatment in the dark, was kept directly under a 16/8 light/dark photoperiod immediately after culture on medium.

2.9 Effects of $AgNO_3$ on adventitious shoot regeneration

Four different combinations of hormones were used in this experiment on NN69 medium, including 1.0 $mg \cdot L^{-1}$ TDZ + 0.5 $mg \cdot L^{-1}$ IBA, 1.0 $mg \cdot L^{-1}$ TDZ + 0.3 $mg \cdot L^{-1}$ NAA, 4.0 $mg \cdot L^{-1}$ BA + 0.3 $mg \cdot L^{-1}$ NAA, and 4.0 $mg \cdot L^{-1}$ BA + 0.5 $mg \cdot L^{-1}$ IBA in each treatment. One $mg \cdot L^{-1}$ $AgNO_3$ was added to all treatments except for the control treatments.

2.10 Statistical analysis

To detect significant differences among treatment levels, data were analyzed by statistical package SPSS data analysis software (version 12) and the significance of the means was tested by Duncan-test at $P < 0.05$ probability level.

3 Results and discussion

3.1 Adventitious shoot regeneration

One to two weeks after leaves were inoculated, they were distorted and swelled, and a little white callus was generated from the wounds (Fig. 1 (a)). Another two weeks later, the callus became yellow and harder. After they were transferred to the light, the callus changed to green and buds were formed (Fig. 1 (b)), and during this period of time the number of buds increased dramatically (Fig. 1 (c)). About 30 d after being transferred to the light, no new buds were formed but the newly grown shoots elongated rapidly, as shown in Fig. 1 (d). Most of the buds emerged from the bottom and middle parts of leaves as in Fig. 1 (e), with few from the leaf tip.

3.2 Effects of basic medium on adventitious shoot regeneration

It was found that basic media might affect pear adventitious shoot regeneration significantly. In this experiment, more than 50% of the leaves regenerated buds induced by NN69, followed by 1/2MS, while full MS was less effective for bud regeneration (Table 2). The concentration of TDZ was changed at two different levels, and the results remained the same. Thus, NN69 was the best basic medium for ‘Zhongli 1’ leaf regeneration among the three kinds of media. This result was in accordance with previous researches. Sun (2000) found that 100% of buds were regenerated from ‘Jinhua’ pear leaves on NN69 medium, while MS could not induce any buds. Likewise, Xu et al. (2002) also reported that NN69 was more effective than 1/2MS or MS on ‘Bayuehong’ pear.

3.3 Effects of plant growth regulators on adventitious shoot regeneration

It can be inferred from Table 3 that the best combination for bud regeneration from the leaves of ‘Zhongli 1’ pear was TDZ and IBA. The regeneration rates of all the combinations from 5 to 8 were higher than 50%, while the other two groups of combinations 1 to 4 and 9 to 16 had

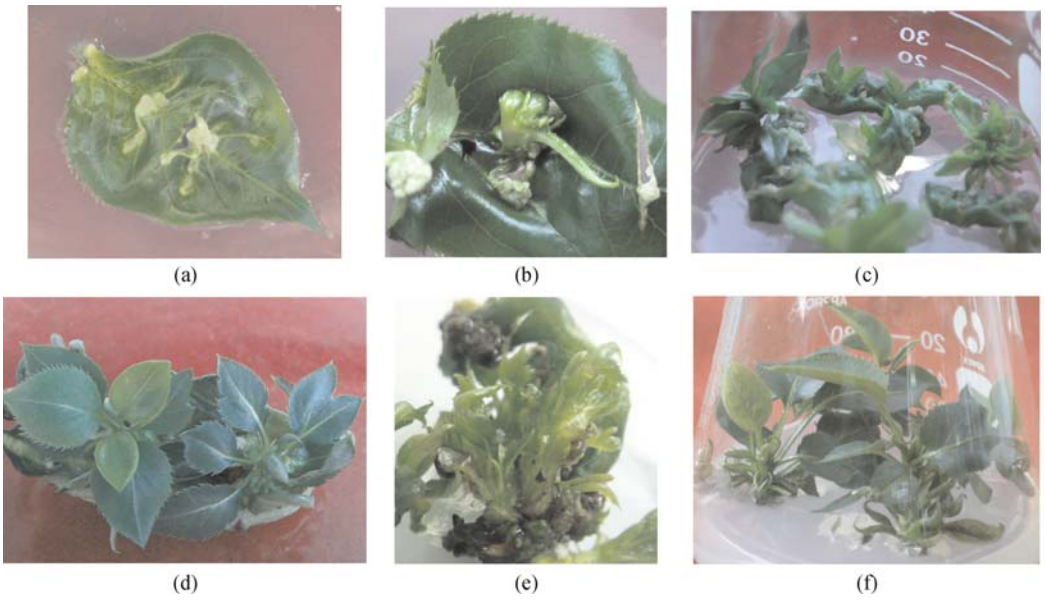

Fig. 1 Procedures of the adventitious shoot regeneration from leaves

Note: (a) – (f) represent formation of white callus from the cut surface of leaves, initial stage of adventitious shoot regeneration, adventitious shoots from leaves, elongation of adventitious shoots on leaves, many adventitious shoots from one leaf, and elongated shoots in SEM, respectively.

Table 2 Different types of media and their effects on adventitious shoot regeneration

basic medium	hormone concentration/mg·L^{-1}	regeneration frequency/%	shoot number per leaf
MS	TDZ 1.0 mg·L^{-1} + IBA 0.5 mg·L^{-1}	0c	0b
1/2MS		13.33bc	0.17b
NN69		56.67a	1.55a
MS	TDZ 2.0 mg·L^{-1} + IBA 0.5 mg·L^{-1}	1.67c	0.02b
1/2MS		26.67b	0.32b
NN69		61.67a	1.37a

Note: Values followed by different small letters are significantly different at 0.05 probability level.

lower regeneration rates. This showed that the combination of TDZ and IBA was better than the combination of BA and NAA. The optimal concentration of TDZ was 1.5 mg·L^{-1}, when the medium was supplemented with TDZ 1.5 mg·L^{-1} and IBA 0.5 mg·L^{-1} (combination 7), 85% mean shoot regeneration frequency was achieved, and on average, 2.72 buds were found on one leaf. Since the 1980s, TDZ, as a new kind of cytokinin, has been widely used in plant tissue culture. The above results indicate that the effect of TDZ was better than that of BA on 'Zhongli 1' pear regeneration. The regeneration frequencies of combinations from 5 to 8 were all higher than those of 1 to 4, while combinations of 13 to 16 got more adventitious shoots than combinations of 9 to 12. Sun et al. (2003) did an experiment on giant duck pear and found similar results. Zhou et al. (2007) also found that different concentrations of TDZ significantly affected 'Hosui' pear leaf regeneration, and if the medium was supplemented with TDZ 2.0 mg·L^{-1} and IBA 0.1 mg·L^{-1}, the regeneration rate of 'Hosui' would reach 87.6%, with a bud number of 2.92 per leaf. However, we observed during the progress of the experiment that the use of TDZ more easily caused hyperhydricity (Fig. 2) than BA, and many adventitious shoots regenerated from the medium added with TDZ became vitrified and could not be used for further proliferation. Zhao et al. (2007) also found the same problem in his research on the 'Dangshansu' pear. Therefore, it is necessary to reduce the quantity of TDZ in the process of regeneration to reduce the vitrification rate of adventitious shoots. IBA and NAA are all commonly used as auxins in pear tissue culture, but the effects may be different depending on different cultivars. Han et al. (2002) studied the effects of IBA and NAA on 'Bayuehong' pear petiole regeneration and they found that IBA was more effective than NAA, which was the same as in our case; while Tang et al. (2005) reported that NAA was

Table 3 Effects of different plant growth regulator combinations on bud regeneration of leaves

combination number	combination of plant growth regulator/mg·L^{-1}				regeneration frequency/%	shoot number per leaf
	BA	TDZ	IBA	NAA		
1	3	–	0.5	–	28.33defgh	0.42def
2	4	–	0.5	–	40.00cdefg	0.62cd
3	5	–	0.5	–	25.00efgh	0.33def
4	6	–	0.5	–	11.67h	0.17f
5	–	0.5	0.5	–	50.00bcd	0.62cd
6	–	1	0.5	–	56.67bc	1.55b
7	–	1.5	0.5	–	85.00a	2.72a
8	–	2	0.5	–	61.67b	1.37b
9	3	–	–	0.3	16.67h	0.20f
10	4	–	–	0.3	20.00gh	0.23ef
11	5	–	–	0.3	21.67fgh	0.25ef
12	6	–	–	0.3	10.00h	0.12f
13	–	0.5	–	0.3	31.67defgh	0.53cde
14	–	1	–	0.3	45.00bcde	0.57cd
15	–	1.5	–	0.3	43.33bcdef	0.75c
16	–	2	–	0.3	30.00defgh	0.58cd

better than IBA on ‘Manyuanxiang’ pear leaf regeneration. Contrary to this result, in our experiment, we found that IBA was better than NAA for ‘Zhongli 1’ regeneration. As shown in Table 3, 0.5 mg·L^{-1} IBA combined with 1.5 mg·L^{-1} TDZ could induce a regeneration rate of 85%, while the regeneration frequency in the medium added with 0.3 mg·L^{-1} NAA + 1.5 mg·L^{-1} TDZ only reached 43.33%. Different cultivars showed differences towards the same hormone; this contradiction may originate from genotypic differences.

Fig. 2 Vitrification of adventitious shoots

3.4 Effects of leaf placement on medium on adventitious shoot regeneration

Table 4 shows that the ways of leaf placement on the medium significantly affected the regeneration frequency. If the abaxial surface of the leaf (leaf back) touched the medium, the regeneration frequency and bud number per leaf were all obviously higher than those of the obverse side of leaf touching the medium, which was in accordance with that of previous researches on other pear cultivars (Sun et al., 2003; Zhou et al., 2007). In our experiment, we observed that most of the adventitious shoots of ‘Zhongli 1’ pear regenerated from the obverse side of the leaf, so if a leaf touched the medium by its adaxial surface, the buds from the obverse side would grow into the medium, then turn around and grow upside, while if the leaf is placed on the medium by its back, the adventitious shoots would grow directly upside. This may be one reason why leaves with their backs touching the medium differentiated more buds. In addition, compared with the compact palisade tissue on the obverse side of leaves, the spongy tissues on the back were looser, and the stomata were bigger (Xu et al., 2002), therefore it was easier for leaves to absorb nutrients and hormones if their abaxial surfaces touched the medium, and eventually promote the regeneration of adventitious shoots.

3.5 Effects of dark culture periods on adventitious shoot regeneration

Our experiment showed that a dark culture significantly affected ‘Zhongli 1’ leaf regeneration. Without treatment in the dark, only 11.67% of ‘Zhongli 1’ leaves regenerated buds, and after 14 to 28 d treatment in the dark, the regeneration frequency was higher, up to 40% to 56.67%, significantly higher than other treatments, and the best treatment was 21 days of dark culture. However, when they

Table 4 Effects of leaf placement ways on medium on adventitious shoot regeneration

ways that leaves put on medium	regeneration frequency/%	shoot number per leaf
abaxial surface (leaf back) touching medium	56.67a	1.55a
adaxial surface (obverse side of leaf) touching medium	28.33b	0.48b

were cultured in the dark for too long, longer than 42 d, the regeneration rate would dramatically drop (Table 5). As was reported, culturing in darkness is necessary for the formation of adventitious shoots, while light is essential for the growth of those buds (Jun et al., 1996). After a proper time of darkness, such as 21 days, nearly all buds form, then light is needed. During this period, if no light were available, buds would wither and fail to grow when later leaves are transferred into the light. This might be the possible mechanism concerning lower regeneration frequency after leaves are cultured for excessively long periods in darkness. Dark culture has the same effect on other pear cultivars, for instance, 'Cuiguan' pear would get a high frequency of bud regeneration from leaves after a 15-day dark culture, and if longer or shorter, the regeneration rate would be decreased (Cao and Chai, 2005). Zhou et al. (2007) also found a 59% bud regeneration rate after 21 days of dark culture in 'Hosui' pear compared with the 30% regeneration rate when it was directly cultured in light.

3.6 Effects of $AgNO_3$ on adventitious shoot regeneration

In our experiment, it was found that in NN69 medium with 4 different combinations of hormones as shown in Table 6, 1.0 $mg \cdot L^{-1}$ $AgNO_3$ significantly increased the pear regeneration frequency and the bud number per leaf of 'Zhongli 1'. Xu et al. (2002) did an experiment on $AgNO_3$ using 'Bayuehong' pear and they found that, 0.1–1.5 $mg \cdot L^{-1}$ $AgNO_3$ could promote its regeneration rate, and 0.5 $mg \cdot L^{-1}$ was the optimal concentration. Zhou et al. (2007) found that the regeneration rate of 'Hosui' leaves could reach 100% on a medium in which 1.0 $mg \cdot L^{-1}$ $AgNO_3$ had been added, while in the control, only 59.5% leaves differentiated buds. Ethylene, as one kind of gas hormone, significantly inhibited the formation of organs and somatic embryos of plants, and finally hindered *in vitro* the leaf regeneration process (Eng et al., 1996); $AgNO_3$ was one type of inhibitor of ethylene. In view of these details, some researchers presumed that the production of ethylene could be refrained, and consequently, shoot regeneration frequency of *in vitro* leaves was promoted after the application of $AgNO_3$ (Sun et al., 1998). However, Zhang and Ling (1995) did not suggest that the mechanism of $AgNO_3$ was to disturb the production of ethylene, but that the existence of Ag^+ prevented the ethylene from disturbing the production progress of polyamines, which are good for the development of somatic embryos and adventitious shoots. Therefore, further studies are needed.

Table 5 Effects of different dark culture periods on adventitious shoot regeneration

dark period/d	regeneration frequency/%	shoot number per leaf
0	11.67c	0.45cd
7	28.33bc	0.58bc
14	41.67ab	1.48a
21	56.67a	1.55a
28	40.00ab	0.87b
42	8.33c	0.20d

Table 6 Effects of $AgNO_3$ on adventitious shoot regeneration

medium	$AgNO_3$/$mg \cdot L^{-1}$	regeneration frequency/%	shoot number per leaf
NN69 + TDZ 1.0 $mg \cdot L^{-1}$ + IBA 0.5 $mg \cdot L^{-1}$	–	56.67a	1.55a
	1.0	78.33b	1.67a
NN69 + TDZ 1.0 $mg \cdot L^{-1}$ + NAA 0.3 $mg \cdot L^{-1}$	–	45.00a	0.57a
	1.0	66.67b	0.88b
NN69 + BA 4.0 $mg \cdot L^{-1}$ + NAA 0.3 $mg \cdot L^{-1}$	–	20.00a	0.23a
	1.0	48.33b	0.67b
NN69 + BA 4.0 $mg \cdot L^{-1}$ + IBA 0.5 $mg \cdot L^{-1}$	–	40.00a	0.62a
	1.0	68.33b	1.00b

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